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EFFECTS OF ACUTE VALPROATE ADMINISTRATION ON CARNITINE METABOLISM IN MOUSE SERUM AND TISSUES

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Abstract—Carnitine concentrations in serum, liver, kidney, muscle and heart were determined 30 min, 2 hr and 4 hr after administration of single 50 mg/kg doses of valproic acid (VPA) or octanoic acid (OTA) of fasting mice. Half an hour post-administration (p.a.) of VPA, free carnitine concentrations were smaller than in controls in serum, liver, kidney and heart. Four hr p.a., the effects of VPA had disappeared from all the carnitine sources, which now had concentrations that were not significantly different from those of controls. The effects of OTA are different from, and sometimes the opposite of, those of VPA, showing that the effects of VPA are specific to it. Hyperammonemia, on the other hand, was greatest 4 hr p.a. of VPA. These findings show that the effect of VPA on carnitine metabolism is immediate but transient, and accordingly suggest that the carnitine deficiency observed in patients under prolonged treatment with VPA-containing anticonvulsants must be due to a more complex mechanism than direct interaction between carnitine and VPA.

Valproic acid (VPA) is useful for treating epilepsy, but reported side effects include hyperammonemia and a Reye-like syndrome [1–3]. It is reported carnitine deficiency secondary upon VPA therapy in children [4] and adult [5], and others [6–9] have confirmed hypocarnitinemia in patients with Reye-like syndrome associated with VPA adminstration.

Rats treated with VPA exhibit hypocarnitinemia and hyperammonemia, and their liver cells microvesicular steatosis and mitochondrial swelling [10–12]; the association between low carnitine and high ammonia concentrations has been attributed to carnitine protecting against hyperammonemia [5, 13]. VPA also reduces blood ketone concentrations in both suckling mice [14] and fasting epileptic children [15], while the effects of a single dose of VPA on the liver of normal infant mice include reductions in the concentrations of free CoA, acetyl-CoA and free carnitine, and a simultaneous rise in the concentration of acyl-CoA esters due to the enzymatic conversion of VPA to valproyl-CoA [16].

In apparent contradiction with the latter results, it has recently been reported that prolonged administration of large doses of VPA to rats causes free carnitine deficiency in serum, erythrocytes and muscle, but not in liver; and that adding L-carnitine supplement to the diet protects against the alteration of carnitine metabolism by VPA administration [17, 18].

In the work described here we aimed to throw some light on the mechanism by which VPA administration alters carnitine metabolism by determining the evolution of the effects of therapeutic doses of VPA on free carnitine and acylcarnitine concentrations in adult mouse serum, liver, kidney, muscle and heart, and comparing them with those of octanoic acid (OTA).

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MATERIALS AND METHODS

Nine 10-mouse lots of male Swiss albino mice weighing 25–30 g and fed a standard diet *ad lib.* were used (3 VPA-treated groups, 3 OTA-treated groups and 3 controls). All were slaughtered after 8 hr fasting; subcutaneous injection of 50 mg/kg of VPA or OTA was performed 30, 120 or 240 min before slaughter in three groups, while the corresponding control groups were given injections of the same quantity of iso-osmotic sodium chloride solution at the same times. The animals were killed by decapitation; blood was collected from the severed neck vessels and centrifuged at 4° to obtain plasma, and liver, kidneys, muscle and heart were removed and frozen in liquid nitrogen pending analysis.

The frozen organs were homogenized in a Potter–Elvejhem homogenizer with four times their fresh weight of 0.5 mol/L Hepes buffer (pH 7.5) containing 10 mmol/L EDTA. Microcentrifuge tubes containing 0.2–0.5 mL of tissue extract were heated in a water bath at 100° for 6 min, cooled in an ice-water bath and centrifuged for 10 min at 12,000 rpm. A volume of 0.2 mL of the supernatant was drawn off, treated with 0.02 mL of 3% H_2O_2 and left for 10 min at room temperature before 0.01 mL of 5×10^4 kilounits/L catalase solution were added to destroy excess H_2O_2. After 30 min the mixture was centrifuged and the concentration of free carnitine in the supernatant was determined.

Free carnitine concentrations in serum and tissue homogenate supernatant were determined using the method of Rodriguez-Segade *et al.* [19]. Total carnitine concentrations (comprising both free and esterified forms) were measured by the same procedure after acylcarnitine had been hydrolysed with

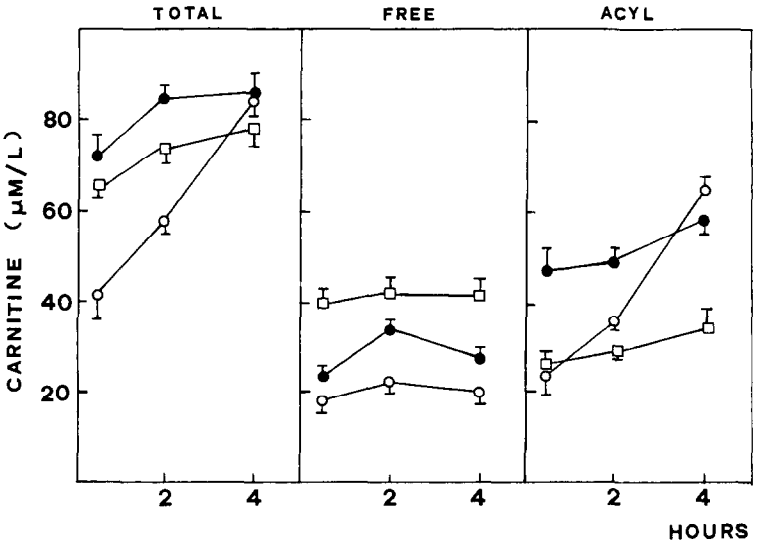

Fig. 1. Free carnitine, acylcarnitine and total carnitine concentrations in mouse serum 0.5, 2 and 4 hr after subcutaneous administration of normal saline (●), 50 mg of VPA per kg of body weight (○) or 50 mg of OTA per kg of body weight (□). Samples were obtained when the mice completed 8 hr without food; carnitine concentrations were determined as described in Materials and Methods. Vertical bars span ±1 SE.

1 mol/L KOH as per McGarry and Foster [20]. Acylcarnitine concentrations were calculated by subtracting free carnitine concentrations measured without alkaline hydrolysis from total carnitine concentrations.

Blood ammonia concentrations were measured by the method based on microdiffusion of ammonia, in which ammonia vapour given off by blood mixed with a dried alkaline buffer (Na_2CO_3–$NaHCO_3$, pH 10.3) diffuses through a polypropylene film that is permeable to gas but not to liquid, and reacts with a pH indicator (Bromocresol Green) whose colour changes proportionally to the concentration of ammonia in the blood. Colour change was measured by the reflectance meter of the Kyoto Daiichikagaku Company's Blood Ammmonia Checker System.

RESULTS

Effect of VPA and OTA on serum carnitine concentrations

Figure 1 shows the post-mortem free carnitine, acylcarnitine and total carnitine concentrations measured in the sera of controls and mice given 50 mg/kg doses of VPA or OTA 30, 120 or 240 min before completion of the 8-hr pre-slaughter fasting. In the VPA groups total carnitine concentrations fell sharply to 41.6 ± 14.4 μmol/L (c.f. controls: 71.6 ± 12.8, $P < 0.001$) in the first 30 min post-administration (p.a.), but had returned to 85.0 ± 10.3 μmol/L 4 hr p.a. The early fall was due largely to a decrease in acylcarnitine concentration, which in 30 min p.a. controls was 47.8 ± 10.4 μmol/L (66.8% of all carnitine) but almost 50% less in VPA-treated mice (24.0 ± 13.3 μmol/L) 30 min p.a. free carnitine concentrations in VPA-treated mice were only just over 25% down on controls (17.6 ± 5.1 as against 23.8 ± 3.1 μmol/L). Four hr p.a., the difference between the free carnitine concentration of VPA-treated mice and controls was similar to that observed after 30 min, with figures of 19.8 ± 5.7 μmol/L for the treated mice and 27.6 ± 5.9 μmol/L for controls, but the acylcarnitine concentration in treated mice was now 2.7 times the 30-min value and 190.9% greater than in controls (65.2 ± 5.6 μmol/L as against 58.8 ± 9.9 μmol/L).

Table 1. Concentration of ammonia in mouse serum 0.5, 2 and 4 hr after subcutaneous administration of normal saline (controls), or 50 mg of VPA or OTA per kg of body weight

Time (hours)	Ammonium (μg/dL) Controls	VPA	OTA
0.5	30.8 ± 12.8	46.2 ± 19.9	31.1 ± 11.2
2	39.5 ± 13.5	51.2 ± 26.0	32.4 ± 17.1
4	37.8 ± 14.5	92.8 ± 34.8*	28.9 ± 10.7

Resuts are given as means ± SD. Values are the mean of 10 mice.
* Significantly different from controls ($P < 0.001$).

The effect of OTA was quite different from that of VPA: in all cases, free carnitine concentrations were significantly greater than in controls, (40.2 ± 5.3 μmol/L after 0.5 hr, $P < 0.001$; 41.8 ± 7.5 μmol/L after 2 hr, $P < 0.05$; 41.3 ± 8.4 μmol/L after 4 hr, $P < 0.001$), while acylcarnitine concentrations were significantly smaller (25.3 ± 5.4 μmol/L after 0.5 hr, 27.9 ± 3.8 μmol/L after 2 hr and 32.0 ± 11.0 μmol/L after 4 hr; $P < 0.001$ in all cases).

Effect of VPA and OTA on blood ammonia concentration

Hyperammonemia set in within 30 min of administration of VPA, and increased progressively to attain a value of 92.8 ± 34.8 μg/L after 4 hr (Table 1).

Table 2. Free carnitine, acylcarnitine and total carnitine concentration in mice liver 0.5, 2 and 4 hr after subcutaneous administration of normal saline or 50 mg of VPA or 50 mg of OTA per kg of body weight

Concn (nmol/g)	Time (hr) 0.5	2	4
Total carnitine			
Saline	506 ± 26	460 ± 37	458 ± 119
VPA	423 ± 43‡	423 ± 23*	513 ± 96
OTA	462 ± 33†	420 ± 46	401 ± 17
Free carnitine			
Saline	433 ± 26	383 ± 32	328 ± 86
VPA	302 ± 35‡	320 ± 31‡	407 ± 69
OTA	436 ± 29	381 ± 36	311 ± 20
Acylcarnitine			
Saline	73 ± 27	77 ± 20	130 ± 43
VPA	121 ± 31†	103 ± 32*	106 ± 35
OTA	38 ± 8†	49 ± 13†	79 ± 14†

Results are given as means ± SD. Values are the mean of 10 mice. Significantly different from controls: *($P < 0.05$), †($P < 0.01$), ‡($P < 0.001$).

There were no significant differences between controls and OTA-treated animals.

Effect of VPA and OTA on carnitine concentration in various organs

Tables 2 to 5 show the effects of VPA and OTA on free carnitine, alcylcarnitine and total carnitine concentrations in mouse liver, kidney, muscle and heart in comparison with values determined in controls. Unlike serum, the tissues analysed exhibited higher acylcarnitine concentrations in VPA-treated mice than in controls 30 min after injection. After 4 hr, however, acylcarnitine concentrations were smaller in treated mice than controls for all tissues except kidney. During the first 2 hr p.a., total carnitine concentration was only significantly depressed in liver (due to the reduction in free carnitine by 30% and 16% after 2 hr). After 4 hr there were no significant differences between treated and untreated mice as regards either of its forms. On a molar basis, all rises in acylcarnitine concentration were equal to corresponding falls in free carnitine concentration.

The effect of OTA differed considerably from that of VPA, especially in liver (Table 2), for which there were no significant differences between the free carnitine levels of controls and OTA-treated animals, while acylcarnitine levels were consistently smaller in the latter ($P < 0.01$). In kidney (Table 3), the effect of OTA on free carnitine was similar to that of VPA, but acylcarnitine levels were higher than in controls after 0.5 hr and lower after 2 and 4 hr. In muscle (Table 4), OTA, like VPA, had no effect on either free or acylcarnitine. In heart (Table 5), OTA and VPA again had no effect after 2 or 4 hr, but after 0.5 hr OTA (but not VPA) significantly reduced total carnitine concentrations ($P < 0.01$) by reducing that of free carnitine ($P < 0.05$), while VPA (but not OTA) significantly increased acylcarnitine concentrations.

Table 3. Free carnitine, acylcarnitine and total carnitine concentration in mice kidney 0.5, 2 and 4 hr after subcutaneous administration of normal saline or 50 mg of VPA or 50 mg of OTA per kg of body weight

Concn (nmol/g)	Time (hr) 0.5	2	4
Total carnitine			
Saline	619 ± 79	661 ± 57	717 ± 117
VPA	694 ± 40†	807 ± 59‡	785 ± 176
OTA	604 ± 72	730 ± 32†	730 ± 79
Free carnitine			
Saline	469 ± 55	523 ± 56	530 ± 99
VPA	435 ± 34	619 ± 42‡	578 ± 120
OTA	434 ± 50	612 ± 30‡	571 ± 62
Acylcarnitine			
Saline	150 ± 36	139 ± 9	187 ± 29
VPA	259 ± 6‡	186 ± 21	207 ± 57
OTA	184 ± 33	119 ± 22*	151 ± 19†

Results are given as means ± SD. Values are the mean of 10 mice. Significantly different from controls: *($P < 0.5$), †($P < 0.01$), ‡($P < 0.001$).

Table 4. Free carnitine, acylcarnitine and total carnitine concentration in mice muscle 0.5, 2 and 4 hr after subcutaneous administration of normal saline or 50 mg of VPA or 50 mg of OTA per kg of body weight

Concn (nmol/g)	Time (hrs) 0.5	2	4
Total carnitine			
Saline	322 ± 26	324 ± 41	399 ± 55
VPA	375 ± 38	349 ± 41	377 ± 114
OTA	296 ± 34	296 ± 39*	363 ± 20
Free carnitine			
Saline	206 ± 33	259 ± 49	250 ± 42
VPA	238 ± 36	250 ± 60	298 ± 54
OTA	205 ± 50	239 ± 40	259 ± 36
Acylcarnitine			
Saline	116 ± 28	66 ± 9	149 ± 94
VPA	138 ± 19*	100 ± 49	79 ± 69
OTA	91 ± 23	57 ± 33	104 ± 28

Results are given as means ± SD. Values are the mean of 10 mice. Significantly different from controls: *($P < 0.05$).

DISCUSSION

It has been reported [4, 5, 9, 21–24] that prolonged treatment with anticonvulsants containing VPA causes hypocarnitinemia in epileptic patients. Similarly, rats administered 500 mg/kg twice daily for 7 days have exhibited a decrease in free carnitine concentration and a rise in acylcarnitine concentration and the acyl/free ratio [17, 18]. In this study, a dose of VPA within the therapeutic range had an immediate effect on carnitine metabolism, producing, within 30 min, significant differences between VPA-treated mice and controls with regards to the free carnitine/acylcarnitine balance in both tissues and serum; there were nevertheless no differences between treated and untreated mice 4 hr p.a. This suggests that the persistent hypocarnitinemia observed in patients was either limited to the bloodstream (samples were taken from these patients before breakfast, and in the present study serum free carnitine concentration 4 hr p.a. was 28% less than in controls) or that carnitine deficiency secondary upon prolonged VPA treatment is caused by a more roundabout metabolic effect than direct interaction between VPA and carnitine.

Table 5. Free carnitine, acylcarnitine and total carnitine concentration in mice heart 0.5, 2 and 4 hr after subcutaneous administration of normal saline or 50 mg of OTA per kg of body weight

Concn (nmol/g)	Time (hr) 0.5	2	4
Total carnitine			
Saline	1236 ± 117	1199 ± 160	1272 ± 45
VPA	1240 ± 180	1224 ± 63	1253 ± 100
OTA	1089 ± 86†	1135 ± 99	1210 ± 210
Free carnitine			
Saline	1105 ± 105	1075 ± 147	1066 ± 138
VPA	1005 ± 118	1108 ± 66	1094 ± 126
OTA	978 ± 106*	957 ± 84	1013 ± 199
Acylcarnitine			
Saline	131 ± 18	123 ± 22	206 ± 95
VPA	235 ± 48‡	161 ± 50	159 ± 68
OTA	113 ± 74	166 ± 66	197 ± 36

Results are given as means ± SD. Values are the mean of 10 mice. Significantly different from controls: *($P < 0.05$), †($P < 0.01$), ‡($P < 0.001$).

VPA is an eight-carbon fatty acid that reacts with carnitine to form the ester valproylcarnitine [25]. Becker and Harris [26] have found that the rise in the concentration of medium-chain acyl CoA esters in the liver of adult rats given VPA injections is due to the accumulation of valproyl-CoA and an ester that appears to be a metabolite of valproyl-CoA. Thurston *et al.* [16] reported that the livers of suckling mice administered single doses of VPA exhibited a sharp fall in the concentration of CoA and a concomitant rise in the concentration of acid-soluble (non-acetyl) acyl-CoA esters, suggesting that valproate had undergone enzymatic conversion to valproyl-CoA and its derivatives. The decrease in free carnitine concentrations and increase in acylcarnitine concentrations observed in various organs in the present study corroborate this hypothesis.

That the effects of VPA on carnitine are specific to VPA is shown by the fact that administration of the same dosage of its physiological isomer, OTA, causes free carnitine concentrations to rise considerably, not to fall as after administration of VPA, while the drop in acylcarnitine concentration brought about by both OTA and VPA is recovered after 4 hr in the case of VPA but not in the case of OTA (Fig. 1). These results are in keeping with resports that the activity of hepatic medium-chain acyl-CoA hydrolase is 10 times greater for octanoyl-CoA as substrate than for valproyl-CoA [27]. Again, Becker and Harris [26] have found that the concentration of medium-chain acyl-CoA in rat hepatocytes is increased 405% by administration of 1 mM VPA as against only 70% when induced by 2 mM OTA.

The reduction in serum free carnitine concentrations associated with prolonged VPA therapy in epileptic patients may be induced in at least two ways. In the first place, it is possible that VPA treatment reduces the production of carnitine from gamma-butyrobetaine in the liver, though Nishida *et al.* [17] found normal free carnitine levels in the livers of rats treated with VPA. Secondly, it is possible that the rate of reabsorption of free carnitine by the kidney is reduced. Matsuda *et al.* [21] found their patients to have increased urinary excretion of both free and acylcarnitine, together with reduced reabsorption of free carnitine and a reduced acylcarnitine/free carnitine clearance ratio, showing a lowering of the renal threshold for free carnitine. The sharp decrease in serum acylcarnitine concentration caused by VPA administration in our own experiments, which was greater than the fall in serum free carnitine concentration, suggests that carnitine was lost in the form of carnitine esters via a "renal leak" in a way similar to that described for organic acidura [28, 29]. The fact that Nishida *et al.* [17] did not observe any fall in serum acylcarnitine concentration may have been due to their having obtained their samples when their animals had spent a night without food after administration of VPA, whereas in those of our experiments in which a marked reduction of acylcarnitine concentrations was observed, VPA was administered just 30 min before the end of 8 hr of fasting. Nishida *et al.* [17] also used rats instead of mice, and VPA doses of 500 mg/kg, 10 times greater than ours.

The hyperammonemia induced by VPA administration had not been recovered from 4 hr p.a. On the contrary, it was at this time that the greatest blood ammonia concentrations were observed (OTA, on the other hand, induced no significant differences in ammonemia with respect to controls). These results are apparently at odds with those of Haidukewych *et al.* [30], who reported that administration of VPA was necessary but not sufficient for increased incidence of venous hyperammonemia in chronic patients, since above-normal ammonia venous ammonia concentrations were found when VPA was given with phenobarbital or with phenobarbital and phenytoin, but not when given alone or with carbamazepine. Further research is necessary to determine whether blood ammonia concentration returns to normal later than 4 hr p.a.

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