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Reversal of the Adverse Chronic Effects of the Unsaturated Derivative of Valproic Acid—2-*n*-Propyl-4-Pentenoic Acid—on Ketogenesis and Liver Coenzyme A Metabolism by a Single Injection of Pantothenate, Carnitine, and Acetylcysteine in Developing Mice

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ABSTRACT. Like treatment with the parent compound valproic acid (VPA), acute and/or chronic treatment with the unsaturated derivative, 2-*n*-propyl-4-pentenoic acid (4-en-VPA), decreased ketogenesis and lowered free CoA, acetyl CoA, and free carnitine levels in the livers of normal developing mice. Concomitantly, there were manifold increases in the content of medium-chain acyl CoA esters (4-en-VPA CoA and 4-en-VPA CoA metabolites). Acute cotreatment of 4-en-VPA–treated animals with pantothenate, carnitine, and acetylcysteine caused significant amelioration of these metabolic aberrations. In animals chronically treated with 4-en-VPA, a single injection of pantothenate, carnitine, and acetylcysteine returned the 4-en-VPA–depressed levels of β-hydroxybutyrate in plasma and free CoA and acetyl CoA in liver to normal. These findings support the hypothesis that VPA- and 4-en-VPA–induced hepatic dysfunction is produced by CoA sequestration rather than by irreversible inhibition by alkylation of the enzymes of fatty acid β-oxidation by reactive intermediates. The findings also support the important but little-known role of carnitine in CoA metabolism—carnitine relieves the inhibition of pantothenate kinase, the rate-controlling first enzyme in the pathway of CoA synthesis by its product, free CoA, and by CoA esters. (*Pediatr Res* 33: 72–76, 1993)

Abbreviations

VPA, valproic acid (2-*n*-propylpentanoic acid)
4-en-VPA, 2-*n*-propyl-4-pentenoic acid
4-PA, 4-pentenoic acid

Inhibition of fatty acid oxidation by VPA is an invariate finding both *in vitro* and *in vivo* (1–8 and many others). Two major mechanisms for this adverse action have been proposed. One relates to the depletion of free CoA in liver due to its sequestration in poorly metabolized valproyl CoA and valproyl CoA derivatives (3, 9). The other hypothesis is that highly reactive unsaturated intermediates of VPA alkylate, and thereby cause irreversible inhibition of, key enzymes of β-oxidation (10, 11).

Gerber *et al.* (12) were among the first to report VPA-associated irreversible hepatic failure in young infants and children. Because of the structural similarity of the unsaturated metabolite of VPA, 4-en-VPA, to two well-known hepatotoxins—4-pentenoic acid and toxic hypoglycin A metabolites (13, 14)—the authors believed that 4-en-VPA played a major role in the pathogenesis of VPA-induced hepatotoxicity. The present study is a test of this hypothesis in normal developing mice. Its results appear to be applicable to an explanation of the mechanism of action of these other hepatotoxins. Because we have found that coadministration of pantothenate and carnitine with VPA ameliorated its adverse effects on ketogenesis and liver CoA metabolism (15), effects of a similar regimen in acutely and chronically 4-en-VPA–treated mice were also examined.

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MATERIALS AND METHODS

Pantothenate (D-pantothenic acid, hemicalcium salt), and N-acetyl-L-cysteine were purchased from Sigma Chemical Co., St. Louis, MO. Solutions of N-acetyl-L-cysteine were neutralized to pH 7.0 with NaOH just before use. 4-en-VPA and sterile 0.9% NaCl were gifts of Abbott Laboratories, Chicago, IL; L-carnitine was a gift of Sigma-Tau, Rome, Italy.

Preparation of Animals. All animal experiments were performed with the highest standards of humane care.

Acute 4-en-VPA Administration and Cotreatment with Pantothenate, Carnitine, and Acetylcysteine. Five litters of 4- to 8-d-old Swiss Webster mice (total 34) were used. The mean age (±SEM) of these mice was 5.2 ± 0.6 d; their mean weight was 3.52 ± 0.22 g. Weight-matched littermates were injected s.c. with 20–30 mg/kg of 4-en-VPA in 20 mL/kg of 0.9%NaCl or an equivalent volume of 0.9% NaCl (controls). Findings in experimental mice receiving 20 or 30 mg/kg of VPA or 4-en-VPA, acutely or chronically, were not significantly different; therefore, results were pooled. Other littermates received 4-en-VPA plus pantothenate (vitamin B_5; CoA precursor), 2 mmol/kg; carnitine, 2.5 mmol/kg; and acetylcysteine, 1 mmol/kg. All animals were killed by decapitation 90 min after injection. Blood was collected from the severed neck vessels and the body was plunged into liquid nitrogen with rapid stirring to effect quick freezing. We have previously determined that liver metabolite levels in neonatal and developing mice of this age frozen in liquid nitrogen were not different from those found in neonatal and developing mice frozen in liquid Freon 12 (CCl_2F_2) at its melting point of 150°C (unpublished data).

Chronic 4-en-VPA Administration and Treatment with Pantothenate, Carnitine, and Acetylcysteine. Two litters of 5- to 7-d-old mice were used for this study (total 19 animals). The mean age of these mice on the final day of the experiment was 10.0 ± 0.4 d; their mean weight was 5.38 ± 0.27 g. The mice received 4-en-VPA (20–30 mg/kg) or 0.9%NaCl as described above, once daily for 5 d. On the last day, some 4-en-VPA–injected littermates were treated with pantothenate, carnitine, and acetylcysteine in the dosage given above. The animals were killed 90 min later. Subsequent methods of procedure were as described above.

Preparation of Plasma and Liver. Blood was promptly centrifuged at 4°C. Plasma was deproteinized with 10 to 20 volumes of 0.5 M perchloric acid. It was not necessary to neutralize the acid extracts for assay of plasma metabolites; aliquots of the acid extract required for fluorometric measurements of β-hydroxybutyrate did not change the pH of the reagent buffer or affect complete recovery of the standard. Liver was dissected free of membranes and visible blood vessels with sharp chisels in a cryostat at −35°C. Perchlorate extracts were prepared in a cold room at −20°C (16). Extracts were stored at −80°C until the time of assay.

Analytical Methods. β-Hydroxybutyrate. The plasma β-hydroxybutyrate level was measured by a fluorometric adaptation of the method of Williamson *et al.* (17).

Free CoA and CoA esters. Free CoA, acetyl CoA, and acyl CoA esters were measured by the cycling procedures of Kato (18) and McDougal and Dargar (19). Medium-chain acyl CoA esters were hydrolyzed by heating a portion of the perchloric acid extract for 60 min at 60°C in the presence of 6 mM DTT and 70 mM 2-amino-2-methyl-1-propanol buffer at pH 9.2 (19). The concentration of medium-chain acyl CoA esters was calculated by subtracting the previously determined concentrations of free CoA and acetyl CoA. The perchloric acid-insoluble precipitate was used for the measurement of long-chain acyl CoA esters.

Free carnitine and carnitine esters. Free carnitine was assayed fluorometrically by measuring the CoA formed (20) from the reaction of carnitine with acetyl CoA in the presence of carnitine acetyltransferase (EC 2.3.1.7). Short- and medium-chain acyl carnitine esters were hydrolyzed by heating a portion of the neutralized perchloric acid extract for 1 h at 37°C in the presence of 150 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; the pH of the solution was adjusted to approximately 13 with 10 N NaOH (21, 22). After neutralization with 5 N HCl, the total acid-soluble carnitine content was measured as described above. The concentration of short- and medium-chain acyl carnitine esters was calculated by subtracting the concentration of free carnitine. The levels of long-chain acyl carnitine esters were similarly determined using the perchloric acid-insoluble precipitate.

Statistical Analysis. The statistical significance of the difference between the means of control and experimental values in the acute and chronic studies was determined by a one-way analysis of variance, followed by Tukey's Honestly Significant Difference test (23) to identify differences between specific pairs of means.

RESULTS

Clinical. There were no apparent clinical side effects of acute or chronic 4-en-VPA administration; the behavior of the experimental animals could not be distinguished from that of their control littermates. At the end of 5 d of 4-en-VPA injections, the weight of the animals was not different from that of control littermates (not shown). At the time of decapitation, the animals in the chronic study were twice the age of those used for the acute study (10 *versus* 5 d) and weighed 53% more.

Effect of Acute 4-en-VPA Alone and 4-en-VPA Plus Pantothenate, Carnitine, and Acetylcysteine on Ketogenesis and Liver CoA and Carnitine Metabolism. Acute administration of 4-en-VPA lowered the plasma β-hydroxybutyrate concentration 51% (Table 1). It should be recalled that because of the high fat content of maternal milk, ketonemia is physiologic in suckling rodents.

In liver, 4-en-VPA caused a 56% drop in the level of free CoA and a 70% decrease in the acetyl CoA content. Concomitantly, there was a 7-fold increase of medium-chain acyl CoA esters [4-en-VPA CoA and 4-en-VPA CoA metabolites (3) and other medium-chain acyl CoA esters]. Seventy percent of the increase in medium-chain acyl CoA could be accounted for by the combined drop in free and acetyl CoA. As we have previously found for VPA (9), 4-en-VPA did not change the content of long-chain acyl CoA esters. There was a nearly 4-fold increase in the acyl CoA:free CoA concentration ratio.

The drop in the free carnitine level was similar to that of free CoA: 52%. There was no change in the level of short- and medium-chain acyl carnitine esters. The long-chain acyl carnitine ester fraction almost tripled. The total carnitine content was unchanged. There was a 2.5-fold increase in the acyl carnitine:free carnitine concentration ratio.

Coadministration of pantothenate, carnitine, and acetylcysteine with acute 4-en-VPA prevented the decrease in plasma β-hydroxybutyrate levels seen with 4-en-VPA alone and caused significant increases in the contents of free CoA and acetyl CoA: 88 and 169%, respectively. These values were still about 17% less than in controls. Pantothenate, carnitine, and acetylcysteine cotreatment induced a further 15% increase in the content of medium-chain acyl CoA esters. The total CoA content increased 31%. Concomitantly, there was a 33% drop in the 4-en-VPA–elevated acyl CoA:free CoA concentration ratio; the 34% decrease in the corresponding acyl carnitine:free carnitine ratio was not statistically significant.

Effect of Chronic 4-en-VPA Alone and Treatment with Pantothenate, Carnitine, and Acetylcysteine on Ketogenesis and Liver CoA Metabolism. Because the hepatotoxic effects of 4-en-VPA might worsen with time or might not have responded as well to treatment with pantothenate, carnitine, and acetylcysteine as in the acute study, the effects of chronic 4-en-VPA administration were examined (Table 2). Chronic 4-en-VPA lowered the plasma β-hydroxybutyrate concentration 38%. The liver free CoA content also fell 38%; acetyl CoA levels decreased 52%. The content of medium-chain acyl CoA esters increased 14-fold. Only one half of the increase could be accounted for by the drop in free CoA and acetyl CoA. The total CoA content increased 29%. The acyl CoA:free CoA ratio increased 5-fold.

Qualitatively and quantitatively, the effects of chronic 4-en-VPA on liver carnitine metabolism were similar to those seen in the acute study. The 54% increase in the content of long-chain acyl carnitine esters did not quite reach statistical significance.

A single coinjection of pantothenate, carnitine, and acetylcysteine on the 5th (last) d of 4-en-VPA injections returned the 4-en-VPA–depressed level of plasma β-hydroxybutyrate to normal. Levels of free CoA and acetyl CoA in liver were now also not different from the control values. The elevated medium-chain acyl CoA ester fraction was not reduced.

Effects of pantothenate, carnitine, and acetylcysteine on carnitine metabolism were similar to those seen in the acute study except that now the 60% reduction of the acyl carnitine:free carnitine ratio induced by the vitamin, carnitine, and acetylcysteine mixture was statistically significant.

DISCUSSION

As was the case for VPA (15), acute coadministration of pantothenate and carnitine ameliorated the ill effects of 4-en-VPA on ketogenesis and liver CoA metabolism in developing mice. Additionally, a single injection of pantothenate, carnitine, and acetylcysteine brought about a reversal of the adverse hepatotoxic effects of chronic 4-en-VPA: plasma β-hydroxybutyrate

Table 1. *Effect of pantothenate, carnitine, and N-acetylcysteine on plasma β-hydroxybutyrate levels and liver CoA and carnitine metabolism in suckling mice treated acutely with 4-en-valproate**

Measurement	Control (n = 10)	4-en-valproate (n = 13)	4-en-valproate + B_5 + CARN + NAC (n = 11)
Plasma (mM; mean ± SEM)			
β-Hydroxybutyrate	0.68 ± 0.04	0.33 ± 0.01	0.58 ± 0.04‡
Liver (μmol/kg; mean ± SEM)			
Free CoA	88.7 ± 5.4	38.8 ± 1.1†	73.1 ± 1.5†‡
Acetyl CoA	43.0 ± 1.6	13.1 ± 0.4†	35.3 ± 0.7†‡
Medium-chain acyl CoA	17.9 ± 2.2	133 ± 4†	153 ± 6†§
Long-chain acyl CoA	27.3 ± 1.2	27.3 ± 1.0	32.0 ± 4.3
Total CoA	178 ± 8	209 ± 7‖	274 ± 8†‡
Acyl CoA/free CoA	1.13 ± 0.09	4.21 ± 0.17†	2.80 ± 0.10†‡
Free carnitine	221 ± 20	107 ± 12†	1145 ± 9†‡
Short- and medium-chain acyl CARN	342 ± 22	358 ± 19†‡	2930 ± 96
Long-chain acyl CARN	32.2 ± 8.5	89.2 ± 5.2‖	373 ± 23†‡
Total carnitine	592 ± 36	554 ± 31	4448 ± 108†‡
Acyl CARN/free CARN	1.72 ± 0.19	4.40 ± 0.46†	2.88 ± 0.09

* Mice were treated as described in Materials and Methods. B_5, pantothenic acid; CARN, carnitine; NAC, N-acetylcysteine.
† p *vs* control, <0.01.
‡ p *vs* 4-en-valproate, <0.01.
§ p *vs* 4-en-valproate, <0.05.
‖ p *vs* control, <0.05.

Table 2. *Effect of pantothenate, carnitine, and N-acetylcysteine on plasma β-hydroxybutyrate levels and liver CoA and carnitine metabolism in suckling mice treated chronically with 4-en-valproate**

Measurement	Control (n = 8)	4-en-valproate (n = 7)	4-en-valproate + B_5 + CARN + NAC (n = 4)
Plasma (mM; mean ± SEM)			
β-Hydroxybutyrate	0.89 ± 0.07	0.55 ± 0.03†	1.01 ± 0.07‡
Liver (μmol/kg; mean ± SEM)			
Free CoA	131 ± 8	80.5 ± 7.9†	112 ± 5§
Acetyl CoA	33.0 ± 1.9	15.9 ± 1.3†	38.6 ± 3.6‡
Medium-chain acyl CoA	9.8 ± 2.9	142 ± 9†	135 ± 6†
Long-chain acyl CoA	27.3 ± 1.2	27.1 ± 0.2	31.9 ± 4.1
Total CoA	190 ± 9	246 ± 3†	317 ± 11†‡
Acyl CoA/free CoA	0.552 ± 0.059	2.61 ± 0.08†	1.85 ± 0.10†‡
Free carnitine	242 ± 15	121 ± 17†	1003 ± 23†‡
Short- and medium-chain acyl CARN	350 ± 40	462 ± 14	1833 ± 224†‡
Long-chain acyl CARN	71.3 ± 6.7	110 ± 10	350 ± 23†‡
Total carnitine	662 ± 45	686 ± 18	3146 ± 262†‡
Acyl CARN/free CARN	1.79 ± 0.23	5.35 ± 0.87†	2.13 ± 0.21‡

* Mice were treated as described in Materials and Methods. Abbreviations are those used in Table 1.
† p *vs* control, <0.01.
‡ p *vs* 4-en-valproate, <0.01.
§ p *vs* 4-en-valproate, <0.05.

levels were returned to normal as were the depressed levels of free CoA and acetyl CoA in liver; only the medium-chain acyl CoA ester fraction remained elevated.

Differences in the levels of some of the metabolites in the control animals used for the acute and chronic studies are to be expected among the seven different litters of mice used for this research. Whether the older age and the greater weight at the time of decapitation of the mice used for the chronic study contributed to the observed differences cannot be said.

Kesterson *et al.* (7) and Granneman *et al.* (8) have compared the *in vivo* effects of chronic VPA and 4-en-VPA administration on liver intermediary metabolism in young rats. But neither the drug dosages, ages, nor species were comparable to the protocol used in the present study.

Supporting a role of 4-en-VPA in VPA-hepatotoxicity is the fact that chronic coadministration of VPA with phenobarbital in developing mice caused a significant worsening of the adverse effects seen in littermates treated with VPA alone (24). Phenobarbital induces the formation of 4-en-VPA from VPA via the microsomal cytochrome P-450 system (25–27). Chronic cotreatment of young rats with VPA and phenobarbital doubled the plasma 4-en-VPA level and induced hepatic steatosis (7, 8). However, liver acetyl CoA levels were not reduced by 4-en-VPA, and inhibition of β-oxidation was considerably less, not more, severe in animals cotreated with phenobarbital and VPA than in animals receiving only VPA (7). The findings suggest that the steatogenic effect of phenobarbital in the livers of VPA-cotreated rats relates to some mechanism other than inhibition of β-oxidation (8). Metabolite changes in the livers of young 4-en-VPA–injected mice suggested a lesser degree of inhibition of β-oxidation than with VPA (28).

The metabolic aberrations induced by chronic 4-en-VPA therapy were not more severe than those seen in the acutely treated animals. The finding may reflect a compensatory or adaptive response. In contrast with acute *in vitro* VPA studies, chronic VPA administration in developing and adult mice and rats induced significant *in vivo* elevations of the hepatic activities of enzymes of β-oxidation, ketogenesis, and amino acid metabolism (29–31). It is interesting that VPA consistently lowered hepatic levels of the substrates and/or cofactors related to these enzymic reactions (3, 5, 7, 9, 32).

As mentioned earlier, based on the structural similarity of 4-en-VPA to 4-PA it was speculated that the hepatotoxicity of VPA might be linked to the formation of 4-en-VPA and further biotransformation to yield highly reactive electrophilic intermediates (10, 11, 33). In rats injected with radioactive VPA or 4-en-VPA and in hepatocytes, radioactivity was associated irreversibly with unidentified liver proteins (10).

4-PA and unsaturated metabolites inhibited 3-keto-acyl CoA thiolase (EC 2.3.1.16) and/or acetoacetyl CoA thiolase (EC 2.3.1.9) in rat heart mitochondria (34–36). However, Osmundsen *et al.* (37) could not demonstrate inhibition of acetoacetyl CoA thiolase (EC 2.3.1.9) by 4-PA in rat liver mitochondria. To our knowledge, there are no published studies of *in vivo* inhibition of liver 3-keto-acyl CoA thiolase or acetoacetyl CoA thiolase by VPA, 4-en-VPA, or other reactive species.

The observed beneficial *in vivo* effects of pantothenate, carnitine, and acetylcysteine in acute or chronic 4-en-VPA–induced hepatotoxicity should perhaps not be surprising. Inhibition of hepatic fatty acid β-oxidation by 4-PA *in vitro* was reversed by the addition of free CoA to the reaction mixture, with or without carnitine (14, 38, 39). Like VPA and 4-en-VPA, 4-PA also lowered levels of free CoA and free carnitine in liver (13, 40), suggesting that its deleterious effect on liver metabolism was due, at least in part, to sequestration of free CoA and carnitine in relatively inert 4-pentenoyl CoA and carnitine esters.

We found equally beneficial effects in VPA-treated mice by cotreatment with pantothenic acid and carnitine with or without cysteine (15). Farrell *et al.* (41) have reported a striking effect of oral acetylcysteine in three children with VPA-induced hepatotoxicity. The beneficial effect was attributed to the action of acetylcysteine to conjugate VPA and reactive metabolites (42). Hepatic hydrolysis of acetylcysteine to liberate cysteine for CoA synthesis could also have contributed to the improvement of these children. Coinjection of acetylcysteine with VPA in our developing mice had no effect on the VPA-induced inhibition of ketogenesis (unpublished data).

The beneficial effect of carnitine in the treatment of organic acidemias is believed to be due to conversion of accumulated CoA esters of these acids via the action of carnitine acyltransferases to their corresponding carnitine derivatives. However, unlike the physiologic organic acids, VPA does not readily react with carnitine acyltransferase in rat liver (3). In VPA-treated mice, coadministration of a 28-fold greater dose of carnitine had no effect on the depressed levels of ketone bodies in the blood and free CoA or acetyl CoA in liver (15). Nor did carnitine cotreatment, with or without pantothenate, lower the greatly elevated medium-chain acyl CoA ester fraction (including valproyl CoA and valproyl CoA metabolites) (15).

Carnitine esters of VPA or VPA metabolites may not readily be formed by human liver either. In children receiving valproate and carnitine, valproyl carnitine accounted for <10% of the total acyl carnitines in urine (43). In another human study, valproyl carnitine esters in urine increased from 0.4% before carnitine supplementation to only 1% after (44).

It is also relevant that valproyl CoA is poorly hydrolyzed by medium-chain acyl CoA hydrolase activity in liver preparations from both control and valproate-fed rats and rabbits (45).

Pantothenate alone increased the levels of free CoA and acetyl CoA in liver after VPA-induced depression but had no effect on the reduced concentration of β-hydroxybutyrate in plasma (ketogenesis) (15). Only after the addition of carnitine with pantothenate did we see stimulation of ketogenesis and further statistically significant increases of free CoA and acetyl CoA in liver. This cooperative action may relate to the important but less well-known role of carnitine in CoA metabolism. In heart, liver, and kidney, pantothenic acid kinase (EC 2.7.1.33), is strongly inhibited by the end product, free CoA and its acyl esters (46–48). Free carnitine deinhibits the CoA-inhibited enzyme (48, 49). The need for the addition of carnitine with pantothenate to increase ketogenesis and to elevate the depressed levels of free CoA and acetyl CoA in the livers of our VPA and 4-en-VPA–treated mice strengthens these observations.

The amelioration or curative effects of coadministration of pantothenate and carnitine seen with VPA- or 4-en-VPA support the CoA depletion theory of valproate hepatotoxicity. Although direct inhibition (by alkylation) of enzymes of β-oxidation and other important enzymes may play a role in the production of VPA-induced hepatotoxicity, no evidence requiring such an explanation was seen in this study. Specifically, treatment with pantothenate, carnitine, and acetylcysteine produced increased levels of acetyl CoA and free CoA in liver and β-hydroxybutyrate in plasma, but it did not reduce the level of the medium-chain acyl CoA ester fraction where toxic 4-en-VPA and derivatives would be expected to be.

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