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L-CARNITINE PROTECTION IN AMMONIA INTOXICATION

EFFECT OF AMINOCARNITINE ON CARNITINE-DEPENDENT METABOLISM AND ACUTE AMMONIA TOXICITY

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Abstract—Intraperitoneal administration of L-carnitine (16 mmol/kg) was reported by O'Connor *et al.* (*FEBS Lett* **166**: 331–334, 1984) to fully protect mice from ammonium acetate given at a dose that kills 100% of untreated controls. Other investigators either have failed to observe protection by L-carnitine or have attributed the increased survival to a nonspecific "osmoprotective effect" of quarternary ammonium compounds. In the present studies we have confirmed the protective effect of L-carnitine in acute ammonia intoxication and have shown that D-carnitine and deoxycarnitine, close structural analogs of L-carnitine, are without protective effect. Although D-carnitine and deoxycarnitine do not support L-carnitine-dependent metabolisms, they are transported into tissues and their solutions are osmotically identical to those of L-carnitine; lack of protection by D-carnitine and deoxycarnitine suggests that metabolic rather than nonspecific osmotic effects account for L-carnitine-mediated protection. Further supporting the importance of L-carnitine-dependent metabolisms, we found that mice exhibited increased sensitivity to ammonium acetate when pretreated with DL-aminocarnitine, acetyl-DL-aminocarnitine or palmitoyl-DL-aminocarnitine, potent inhibitors of the carnitine acyltransferases. Interestingly, intraperitoneal injection of hyperosmotic solutions of sodium chloride or sucrose did afford significant protection against subsequently administered ammonium acetate. This phenomenon, which may be due to interference with ammonium acetate uptake from the peritoneal cavity or to reduction of cerebral edema by increased plasma osmolarity, apparently does not play a major role in L-carnitine-mediated protection since, as noted, hyperosmotic D-carnitine and deoxycarnitine solutions were not protective.

In 1984, Grisolia and coworkers reported that intraperitoneal injection of L-carnitine (16 mmol/kg) completely protected mice from ammonium acetate given 30 min later at a dose that killed all control mice [1–3]. Although urea synthesis is stimulated by L-carnitine, that effect alone could not explain the protection since L-carnitine is also protective in mice made hyperammonemic by administration of urease. In the urease model of chronic ammonia intoxication, L-carnitine-treated mice survived with blood ammonia levels that would be lethal in mice not given L-carnitine [1]. The results reported suggested that L-carnitine or a metabolic process dependent on L-carnitine has a direct effect on ammonia metabolism or detoxification or both.

L-Carnitine is necessary for the transport of long-chain fatty acids into the mitochondrial matrix for β-oxidation, and, within the matrix, L-carnitine accepts acyl groups from acyl-CoAs (mainly acetyl-CoA) to release CoASH, a cofactor required for both β-oxidation and citric acid cycle activity [4]. L-Carnitine can thus stimulate mitochondrial synthesis of acetyl-CoA, NADH, and ATP. While these pathways do not directly involve ammonia, ATP is required for ammonia detoxification by the urea cycle in liver and by glutamine synthetase in brain. Although these and other associations between L-carnitine and ammonia metabolism are suggestive, the biochemical basis of L-carnitine-mediated protection in acute ammonia intoxication remains undefined.

Some investigators have been unable to confirm protection by L-carnitine or have suggested that the effect is nonspecific. Deshmukh and Rusk [5] reported that neither L-carnitine nor physiological saline affords significant protection to mice given ammonium acetate. On the other hand, Kloiber *et al.* [6] reported that L-carnitine affords full protection from ammonium acetate, but found that other quarternary amines and saline are also protective. Treatment with trimethylamine *N*-oxide, choline, betaine, or physiological saline allowed 100, 87, 67 or 33%, respectively, of treated mice to survive a subsequent challenge by ammonium acetate given at a dose that killed all control mice. Since none of the tested compounds share the metabolic activities of L-carnitine, Kloiber *et al.* [6] suggested that quarternary amines including L-carnitine have an "osmoprotective" effect that reduces ammonia-mediated membrane damage. Siliprandi and coworkers [7, 8] also suggested that L-carnitine-mediated protection is unrelated to its effects on fatty acid metabolism. They showed that ammonium salts depress mitochondrial respiration and phosphorylation by diverting α-ketoglutarate out of the citric acid cycle. L-Carnitine prevented the swelling characteristic of such metabolically compromised mitochondria by an unidentified mechanism that was believed by the authors to be independent of fatty acid oxidation [7, 8].

The present studies examined the effects of L-carnitine, L-carnitine antagonists, and metabolically inert L-carnitine analogs on acute ammonia intoxication in mice. The results indicate that the protective

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effect of L-carnitine is strongly dependent on its specific metabolic reactions. We also found, however, that physiological saline and concentrated solutions of NaCl or sucrose afforded significant protection from ammonia intoxication, a result suggesting that administration of L-carnitine solutions may have an additional nonspecific protective effect. An abstract reporting these results has appeared [9].

MATERIALS AND METHODS

Male Swiss Webster mice (25–30 g) were obtained from Hilltop Laboratories, Philadelphia, PA, and were fed standard laboratory chow *ad lib.* L-Carnitine, free base, was obtained from Chemical Dynamics, South Plainfield, NJ. DL-Aminocarnitine, acetyl-DL-aminocarnitine, and palmitoyl-DL-aminocarnitine were synthesized as described [10, 11]. All other reagents were from Sigma, St. Louis, MO.

Ammonium acetate solutions of about 0.8 M were prepared daily by weight and were adjusted to pH 8.0 with NH_4OH; the exact ammonia concentration (0.75 to 0.85 M) was determined using glutamate dehydrogenase [12]. Solutions were kept tightly sealed and were used on subsequent days only if reassay showed no change in ammonia content. The LD_{100} of ammonium acetate was determined by giving otherwise untreated mice doses of 11–15 mmol/kg by intraperitoneal injection and observing the mice for behavioral changes and death. Mice surviving >60 min invariably recovered completely. For reasons that were not determined, the LD_{100} of ammonium acetate decreased from 15 to 13 mmol/kg during the course of these and related studies; the LD_{100} was therefore determined as part of each set of experiments and is reported in the table and figure legends.

Statistical analyses were carried out using version 4.1 of SYSTAT (SYSTAT, Inc., Evanston, IL); differences were taken as significant when $P < 0.05$. The specific statistical tests used are indicated in the table and figure legends.

RESULTS

Mice administered ammonium acetate exhibited characteristic symptoms of ammonia intoxication ranging from lethargy and shivering at lower doses to convulsions, coma and death at higher doses. Onset of symptoms was rapid (2–5 min), and death or complete recovery generally occurred within 20 min.

Intraperitoneal injection of L-carnitine (16 mmol/kg) completely protected mice from ammonium acetate given intraperitoneally 30 min later at the usual LD_{100} (15 mmol/kg). Doses of L-carnitine as low as 1 mmol/kg offered significant protection (Table 1). These results accord well with those of O'Connor *et al.* who reported 0, 60, 70, 80, and 100% survival in mice given 0, 1, 2, 8 and 16 mmol/kg of L-carnitine 30 min prior to an LD_{100} dose of ammonium acetate (12 mmol/kg in their studies) [1, 3]. Since others [4] have reported difficulty reproducing the results of O'Conner *et al.*, it may be noted that we occasionally observed "atypical" responses to both ammonium acetate and L-carnitine. Thus, we have seen no indication of toxicity in otherwise untreated mice given ammonium acetate at the usual LD_{100}, and, less frequently, we found that L-carnitine (16 mmol/kg) neither prevented nor delayed death from ammonium acetate. Since such responses could not be predictably elicited, they have not been systematically examined; uncontrolled nutritional factors [13] or anomalous distribution of the injection volume within the abdomen may be responsible. Although "atypical" responses were easily recognized from the unusual physiological response elicited, data from such animals has *not* been selectively excluded from the tables, figure, or statistical analyses.

The effect of L-carnitine was relatively short-lived; at a dose of 16 mmol/kg, significant protection was observed only when the interval between carnitine and ammonium acetate injection was 15–150 min (Fig. 1). Subcutaneously administered L-carnitine was not protective; 0 of 5 mice given L-carnitine (16 mmol/kg) by subcutaneous injection survived ammonium acetate (15 mmol/kg) given intraperitoneally 60 min later. This finding accords with the report of O'Connor *et al.* that L-carnitine is less protective when given subcutaneously, intravenously or intramuscularly rather than intraperitoneally [14].

The short duration of L-carnitine-mediated protection and the requirement for intraperitoneal injection suggested that the protective effect might be due to nonspecific interference with ammonia uptake from the peritoneal cavity. To explore this possibility, mice were given 1.24 M NaCl at several doses and were challenged with ammonium acetate (LD_{100}, 15 mmol/kg) 30 min later. As shown in Table 1, NaCl was markedly protective at doses of 2, 8 and 16 mmol/kg; its protective effect was not significantly inferior to that of L-carnitine ($P = 0.091$). Sucrose (1.24 M, 16 mmol/kg) also afforded full protection from ammonium acetate given 30 min later; 10 of 10 mice survived. Because 1.24 M NaCl and sucrose are not isoionic and/or isoosmotic with 1.24 M L-carnitine, we also determined the protective effect of deoxycarnitine (4-trimethylammonium-butyrate) and D-carnitine, close structural and charge distribution analogs of L-carnitine that are not carnitine acyltransferase substrates. Deoxycarnitine (1.24 M) had no protective effect at any concentration examined (Table 1). Similarly, 1.24 M D-carnitine (16 mmol/kg) had no significant effect on the toxicity of ammonium acetate (15 mmol/kg) given 30 min later; 8 of 10 D-carnitine-treated mice died.

DL-Aminocarnitine and its acyl derivatives are potent inhibitors of the carnitine acyltransferases involved in long-chain fatty acid transport (i.e. carnitine palmitoyltransferase) and in buffering of the acetyl-CoA/CoASH ratio (i.e. carnitine acetyltransferase) [10, 11]. DL-Aminocarnitine (0.2 mmol/kg) lowered the LD_{100} of ammonium acetate from 15 to 13 mmol/kg and significantly increased its toxicity (Table 2). Acetyl-DL-aminocarnitine (0.2 mmol/kg) and palmitoyl-DL-aminocarnitine (0.1 mmol/kg) also significantly increased the toxicity of subsequently administered ammonium acetate (Table 2).

Mitochondrial β-oxidation of octanoate is not dependent on carnitine palmitoyltransferase, and

Table 1. Concentration dependence of the protective effect of L-carnitine and metabolically inert compounds

Compound injected	Percent survival (mice surviving/mice treated) Compound dose (mmol/kg) 0	1	2	8	16
L-Carnitine	0 (0/20)	30 (3/10)*	50 (5/10)†	90 (9/10)‡	100 (14/14)‡
Deoxycarnitine§		20 (1/5)	0 (0/5)	20 (1/5)	20 (1/5)
Sodium chloride‖		20 (2/10)	30 (3/10)*	70 (7/10)‡	90 (9/10)‡

The compounds indicated (1.24 M neutral solutions in saline) were given i.p. at the doses indicated. Thirty minutes later, 0.8 M ammonium acetate, pH 8.0 (15 mmol/kg, i.p.), was given, and survival of the animals was monitored.

*†‡ The effect on survival of specific treatments was compared to survival following ammonium acetate alone (i.e. Compound dose = 0) using Fisher's Exact Test: *$P < 0.04$; †$P < 0.005$; ‡$P < 0.001$.

§‖ The overall effect of L-carnitine on survival was compared to that of deoxycarnitine and sodium chloride using the Mantel–Haenszel Chi Square Test: §$P < 0.001$ and ‖$P = 0.091$.

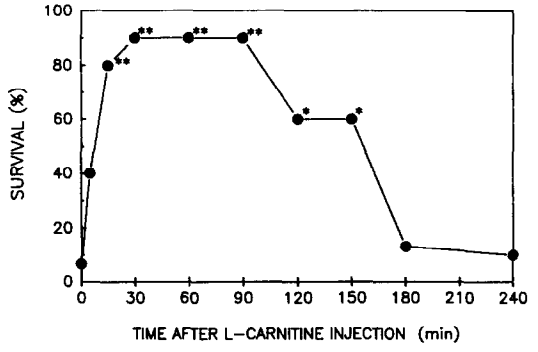

Fig. 1. Effect of the interval between L-carnitine and ammonium acetate administration on survival. Both L-carnitine (16 mmol/kg) and ammonium acetate (13 mmol/kg, LD_{100}) were given by intraperitoneal injection. Values shown are the means of two or three experiments in which groups of 5 mice were tested at the times indicated. Control mice (time = 0) received no L-carnitine. Key: *$P < 0.01$ vs control; and **$P < 0.001$ vs control.

metabolism of [^{14}C]octanoate to $^{14}CO_2$ was shown previously not to be inhibited by aminocarnitine [11]. Administration of octanoate to aminocarnitine-treated mice should thus replete intramitochondrial stores of acetyl-CoA, NADH and ATP depleted by inhibition of long-chain fatty acid oxidation. As shown in Table 2, DL-aminocarnitine had no effect on ammonium acetate toxicity if octanoate was also given ($P = 0.933$ vs untreated controls). Octanoate alone was modestly but significantly ($P < 0.001$) protective against ammonium acetate intoxication; in separate studies where the LD_{100} of ammonium acetate was 13 mmol/kg, octanoate (5 mmol/kg) increased the LD_{50} of ammonium acetate from about 11 to 12 mmol/kg but did not significantly change the toxicity of ammonium acetate at 13 mmol/kg (not shown).

Physiological saline (16 mmol/kg) protected against ammonium acetate intoxication but was less effective than L-carnitine (Table 2). Note that the volume of saline injected intraperitoneally was large in these studies (i.e. 2.6 mL/25 g mouse); physiological saline injected in the volumes used with 1.24 M L-carnitine (i.e. ≤0.32 mL/25 g mouse) was not protective (not shown).

DISCUSSION

In 1984, Grisolia and coworkers reported that L-carnitine-treated mice can survive blood ammonia levels that would be lethal in untreated mice [1]. Since ammonia freely crosses the blood–brain barrier and since ammonia toxicity is manifested primarily in the central nervous system, we initially expected that L-carnitine would directly affect brain ammonia or energy metabolism. Studies with rats showed, however, that L-carnitine-mediated protection does not correlate temporally with brain carnitine levels; brain carnitine increases for 24 hr following L-carnitine injection, but its protective effect is lost after 2.5 hr [15]. This result and the appearance of reports challenging the reproducibility and specificity of protection by L-carnitine in ammonia toxicity [5–8] prompted the present studies.

Our results clearly confirm the main observations of O'Connor and coworkers [1–3]. With respect to contrary reports by others, we note that demonstration of L-carnitine-mediated protection requires careful control of experimental conditions; the concentration of the ammonium acetate administered must be accurately known, and the dose given must not exceed the "minimal" LD_{100}. In our studies the LD_{100} was determined as part of every experiment and was reproducible and constant for groups of mice purchased and maintained together. The LD_{100} did, however, decrease from 15 to 13 mmol/kg of ammonium acetate over a period of several months; such a change, if not detected, could easily obscure the protective effect of L-carnitine.

The finding that subcutaneous L-carnitine was not protective and that intraperitoneal NaCl and sucrose solutions were protective initially suggested that nonspecific effects may play an important role in the protection seen with intraperitoneally injected L-carnitine. Intraperitoneal administration of hyperosmotic solutions may interfere with ammonium

Table 2. Effects of carnitine, carnitine antagonists and sodium chloride on acute ammonia toxicity

Compound injected	Percent survival (mice surviving/mice treated)				
	0.8 M Ammonium acetate dose				
	11 mmol/kg	12 mmol/kg	13 mmol/kg	14 mmol/kg	15 mmol/kg
None	100 (10/10)	80 (8/10)	45 (5/11)	30 (3/10)	0 (0/20)
L-Carnitine*† (16 mmol/kg)				100 (10/10)	100 (14/14)
DL-Aminocarnitine* (0.2 mmol/kg)	50 (5/10)	30 (3/10)	0 (0/10)		
DL-Aminocarnitine (0.2 mmol/kg) + Octanoate‡ (5 mmol/kg)	78 (7/9)	90 (9/10)	40 (4/10)	50 (5/10)	0 (0/8)
Acetyl-DL-aminocarnitine§ (0.2 mmol/kg)	80 (8/10)	30 (3/10)	20 (2/10)		
Palmitoyl-DL-aminocarnitine* (0.1 mmol/kg)	67 (10/15)	27 (4/15)	13 (2/15)		
Saline* (16 mmol/kg)	100 (5/5)	100 (5/5)	100 (4/4)	80 (4/5)	50 (2/4)

L-Carnitine and physiological saline were given i.p. 30 min prior to ammonium acetate (i.p.); DL-aminocarnitine and its acyl derivatives were given by subcutaneous injection 6 hr before ammonium acetate. Octanoate was given i.p. 1 hr prior to ammonium acetate. L-Carnitine, DL-aminocarnitine, acetyl-DL-aminocarnitine, palmitoyl-DL-aminocarnitine, and sodium octanoate were given as 1.24, 0.2, 0.2, 0.1, and 0.15 M solutions, respectively.

*†‡§ Significance was evaluated using the Mantel–Haenszel Chi Square Test; possible correlations between treatment (Compound injected) and outcome (Survival) were thereby examined across the range of ammonium acetate doses used: *$P < 0.001$ and §$P < 0.005$ comparing survival of treated mice to survival of control mice (i.e. Compound injected = None). †$P < 0.005$ vs survival of mice given saline. ‡$P < 0.001$ vs survival of mice given aminocarnitine alone; survival was not significantly different from control mice ($P = 0.933$).

acetate uptake from the peritoneum by simple dilution or by creating an osmotic imbalance that draws physiological fluids into the abdomen causing further dilution. If administration of such solutions also increases plasma osmolarity, such an increase may beneficially prevent or decrease the osmotic swelling of brain astrocytes that is postulated to occur from the intracellular accumulation of glutamine in hyperammonemic states [16]. It is likely that some or all of these effects account for the protection afforded by intraperitoneal injection of NaCl or sucrose in mice subsequently administered ammonium acetate.

Although nonspecific effects appear to account for the protection afforded by NaCl and sucrose solutions, the finding that intraperitoneal injection of hyperosmotic deoxycarnitine and D-carnitine solutions was not protective against subsequently administered ammonium acetate indicates that similar nonspecific protection by carnitine-like compounds is weak or non-existent. Because deoxycarnitine and D-carnitine are isosteric and isoionic with L-carnitine and yield osmotically identical solutions, we conclude that nonspecific osmotic effects or simple dilution do not play a primary role in L-carnitine-mediated protection from the toxicity of ammonium acetate.

The studies with DL-aminocarnitine and its acyl derivatives provide strong evidence that carnitine acyltransferase-dependent metabolisms do contribute importantly to survival in acute ammonia intoxication. Reversal of the effect of aminocarnitine by octanoate indicates specifically that increased fatty acid oxidation contributes to survival. In this regard it is notable that hepatic accumulation of L-carnitine is required to support the increased β-oxidation seen in fasting ketogenesis [17] and that both ketogenesis and chronic feeding of ammonium salts decrease fat storage [17, 18]. If acute ammonia intoxication also mobilizes fatty acids, L-carnitine injection may provide the carnitine necessary for increased hepatic fatty acid oxidation; increased β-oxidation could, in turn, provide energy for urea synthesis. In all tissues, increased concentrations of L-carnitine may be protective by shifting the carnitine palmitoyltransferase equilibrium toward acyl-L-carnitines, thus diminishing the accumulation of membrane-damaging and enzyme-inhibiting fatty acyl-CoAs.

There is considerable evidence that ammonia intoxication causes an energy deficit in the brain ([19] and references therein). Ammonia-mediated diversion of substrates (e.g. α-ketoglutarate) from the malate-aspartate shuttle appears to be a major factor in the decreased availability of energy from aerobic glycolysis in the ammonia-intoxicated brain [19]. We have therefore also considered the possibility that hepatic ketone body synthesis, known to be stimulated by L-carnitine [20], may sustain brain energy needs during ammonia intoxication. Although we found that β-hydroxybutyrate injection was protective in acute ammonia intoxication, we have not been able to demonstrate an effect of L-carnitine on blood ketone body levels in mice given ammonium acetate (Ohtsuka Y and Griffith OW, unpublished results). Although the precise

biochemical mechanisms accounting for L-carnitine-mediated protection in acute ammonia intoxication remain to be defined, the present studies provide the first evidence that carnitine acyltransferases and the L-carnitine-dependent metabolisms catalyzed by these enzymes contribute importantly and specifically to survival. Nonspecific effects due to injection of hyperosmotic L-carnitine solutions may also have a protective role, but such effects appear to be less important.

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