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COMPARISON OF THE EFFECTS OF L-CARNITINE, D-CARNITINE AND ACETYL-L-CARNITINE ON THE NEUROTOXICITY OF AMMONIA

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Abstract—Although L-carnitine has been reported to have protective effects against ammonia toxicity, conflicting results have also been presented and the mechanisms underlying the protection, if any, are not clear. In the present study, we examined the effects of L-carnitine, D-carnitine and acetyl-L-carnitine on the neurotoxicity of ammonia. Administration of ammonium acetate (15 mmol/kg) to mice caused seizures, elevation of blood ammonia and urea concentrations, and marked alterations of brain energy metabolites. Pretreatment with either L-carnitine, D-carnitine or acetyl-L-carnitine reduced the frequency of the seizures, prolonged the time until the first fit, lowered the levels of ammonia in the blood and brain, and suppressed the alterations of brain energy metabolites caused by hyperammonemia. There was no significant difference between L- and D-carnitine in the potency to inhibit the seizures. In addition, there was no difference between the two chemicals in the potency to decrease the ammonia contents in the blood and brain, or to suppress the alterations of energy metabolites in the brain. When compared with L-carnitine, however, acetyl-L-carnitine better preserved ATP in the brain, while it lowered ammonia in the blood and brain less markedly. These results show that L-carnitine and its analogues do have the potential to suppress the neurotoxicity of ammonia. Moreover, the results suggest that the protective effects of carnitine against the toxicity of ammonia are systemic, that the action of acetyl-L-carnitine may differ from that of L- or D-carnitine, and that the "classical" function of carnitine is not the sole mechanism underlying the suppression of the neurotoxicity of ammonia.

The increase of the ammonia level in the blood or nervous tissue is believed to play a major role in the genesis of dysfunction of the brain in conditions such as hepatic failure, inborn errors of urea cycle function, organic acidemia and Reye's syndrome [1]. Although the production of ammonia in the body (in the intestine) may be reduced by administration of lactulose or antibiotics [1, 2], the removal of ammonia from the body or the counteraction of the neurotoxicity itself seems essential for the treatment of encephalopathy associated with hyperammonemia.

O'Connor *et al.* [3, 4] reported that pretreatment with L-carnitine (β-hydroxy-γ-*N*-trimethylaminobutyrate) reduces the mortality of mice administered a lethal dose of ammonium acetate. Although the protective effects of L-carnitine from ammonia-induced mortality have been seen by other investigators [5–8], contradictory results have also been presented [9, 10]. Moreover, the mechanism by which it suppresses the neurotoxicity of ammonia, if any, has not been clarified. To obtain information about this mechanism, it is necessary to compare the effects of L-carnitine and its analogues on ammonia toxicity, especially in the brain because the major target organ in hyperammonemia is the brain [1].

We found that administration of 15 mmol/kg of ammonium acetate to mice induces seizures and impairs brain energy metabolism [11], and that pretreatment with L-carnitine (10 or 20 mmol/kg) suppresses the seizures and biochemical alterations of the brain caused by hyperammonemia [12]. In the present study, we compared the effects of D-carnitine and acetyl-L-carnitine with those of L-carnitine on the seizures, levels of ammonia and brain energy metabolites in mice with acute hyperammonemia.

MATERIALS AND METHODS

Chemicals. L-Carnitine (inner salt) and D-carnitine (inner salt) were purchased from the Sigma Chemical Co. (St. Louis, MO). Acetyl-L-carnitine (betaine) was a gift from the Earth Chemical Co. (Hyogo, Japan). All other chemicals were reagent grade.

Animal treatment. Male ddY mice weighing 30–35 g, fed a standard chow *ad lib.*, were used. The doses of ammonium acetate and carnitine injected were the same as in our previous experiments [12]. Animals were divided into five groups and injected i.p. with two solutions 30 min apart: group I (N = 14), injection with saline (0.85% NaCl) followed by a second saline injection; group II (N = 15), saline and then 15 mmol ammonium acetate/kg; group III (N = 14), 20 mmol L-carnitine/kg and 15 mmol ammonium acetate/kg; group IV (N = 13), 20 mmol D-carnitine/kg and 15 mmol ammonium acetate/kg;

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group V (N = 14), 20 mmol acetyl-L-carnitine/kg and 15 mmol ammonium acetate/kg.

L-Carnitine, D-carnitine and acetyl-L-carnitine were dissolved in saline as 20% (w/v), 20% (w/v) and 25% (w/v) solutions, respectively. Ammonium acetate was dissolved in saline as a 0.8 M solution. Ten minutes after the injection of ammonium acetate or the second saline solution, the mouse was immersed quickly in liquid nitrogen for 5 min and kept at −80° until the extraction. Some mice in each group were killed by decapitation for the collection of blood from cervical blood vessels.

Observation of seizures. The behavior of each mouse was recorded with a Canon VM-E1 videotape recorder for 10 min after the injection of ammonium acetate. The time until the first seizure and the number of seizures were analyzed later.

Treatment of blood. Blood was processed immediately after its collection. An aliquot (50 μL) was deproteinized for the determination of ammonia, and the remainder was centrifuged to obtain the plasma for the determination of urea. Both samples were stored at −120° until analysis.

Preparation of tissue extracts. The frozen brain was chiseled out, weighed, and powdered in a mortar chilled with liquid nitrogen. The powder was extracted with 4 vol. of 3.0 M perchloric acid at −15° using an all-glass Potter–Elvehjem homogenizer, and then diluted to 1.0 mL/75 mg tissue with 1 mM EDTA at 0°. The mixtures were centrifuged at 5000 *g* for 30 min at 0°, and the supernatants were neutralized with 2.0 M $KHCO_3$. After being centrifuged at 3000 *g* for 15 min at 0° to remove the precipitated $KClO_4$, the supernatants were stored at −120° until analysis [12].

Biochemical determinations. The concentrations of ammonia [13], phosphocreatine (PCr*) [14], creatine [14], ATP [14], ADP [14], AMP [14], glucose [15], pyruvate [13] and lactate [14] in the brain extracts, and urea [13] in the plasma were all determined enzymatically. Brain energy charge potential (ECP) was calculated by the method of Atkinson [16]; ECP = (ATP + 1/2 ADP)/(ATP + ADP + AMP). The concentration of blood ammonia was assayed by the method of Okuda and Fujii [17].

Statistics. The data presented are from more than six mice in each group that survived for 10 min after the injection of ammonium acetate. All values are expressed as means ± SEM. Statistical analyses of means between control (group I) and untreated animals (group II) were determined by Student's *t*-test or Welch's *t*-test. Comparisons of means between untreated (group II) and carnitine-treated animals (groups III, IV or V) were examined by one-way analysis of variance (ANOVA) followed by Dunnett's test. Comparisons of means among carnitine-treated animals (groups III, IV and V) were examined by one-way ANOVA followed by Tukey's test. Statistical analyses of the time until the first seizure and the number of seizures were done by the Kruskal–Wallis test followed by Tukey's test. $P < 0.05$ was considered as statistically significant.

* Abbreviations: PCr, phosphocreatine; and ECP, energy charge potential.

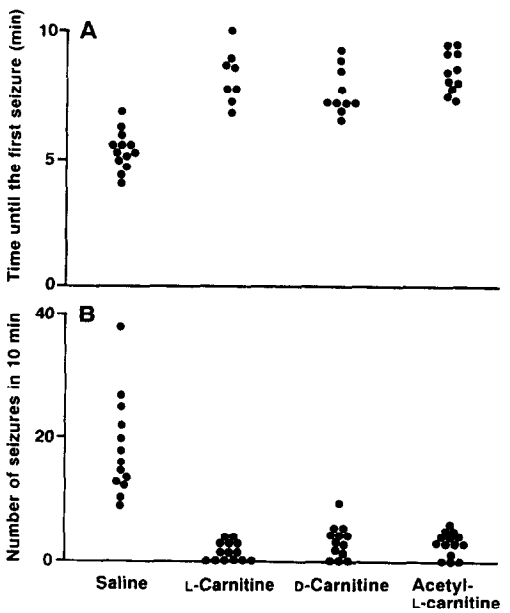

Fig. 1. Effects of L-carnitine, D-carnitine and acetyl-L-carnitine on the time until the first seizure (min) (A) and the number of seizures in 10 min (B). Each mouse was injected with saline solution containing no carnitine, 20 mmol L-carnitine/kg, 20 mmol D-carnitine/kg or 20 mmol acetyl-L-carnitine/kg. Thirty minutes later, the mouse was injected with 15 mmol ammonium acetate/kg and observed (recorded on a videotape) for 10 min. The mice that developed no seizures within 10 min (six in the L-carnitine-treated group; three in the D-carnitine or acetyl-L-carnitine-treated group) are not shown in (A).

RESULTS

Effects on seizures. Two (13.3%) out of 15 untreated animals (group II) died within 10 min after the injection of ammonium acetate. No mice injected with carnitine (groups III, IV and V) died within 10 min after the injection.

All mice injected with ammonium acetate showed a decrease of spontaneous activities and responses to painful stimuli. Within 7 min, all mice in group II developed short clonic seizures. The time until the first seizure was prolonged significantly ($P < 0.01$, Fig. 1A) in animals treated with L-carnitine (group III), D-carnitine (group IV) or acetyl-L-carnitine (group V) compared with group II. The number of seizures in groups III, IV or V was also reduced significantly ($P < 0.01$, Fig. 1B). There were no statistically significant differences in either the time until the first seizure or the number of seizures among carnitine-treated animals.

Effects on levels of ammonia and energy metabolites in the brain. Administration of ammonium acetate caused a marked elevation of ammonia concentration in group II (Table 1). When compared with group I, the levels of PCr, ATP, ECP and glucose decreased significantly ($P < 0.05$), whereas ADP, AMP, lactate and the lactate/pyruvate ratio increased ($P < 0.05$). On the other hand, in carnitine-treated animals

Table 1. Effects of L-carnitine, D-carnitine and acetyl-L-carnitine on brain ammonia and energy metabolite concentrations in mice with hyperammonemia

	Control (group I)	Saline (group II)	L-Carnitine (group III)	D-Carnitine (group IV)	Acetyl-L-carnitine (group V)
Ammonia (μmol/g)	0.38 ± 0.05	5.09 ± 0.12*	2.24 ± 0.14†	2.82 ± 0.05†	3.16 ± 0.33†‡
PCr (μmol/g)	3.16 ± 0.12	2.49 ± 0.11*	3.64 ± 0.13†	3.11 ± 0.21†	3.69 ± 0.11†§
ATP (μmol/g)	2.68 ± 0.09	2.38 ± 0.07*	2.57 ± 0.08	2.48 ± 0.07	2.87 ± 0.08†‡§
ADP (μmol/g)	0.494 ± 0.019	0.618 ± 0.035*	0.456 ± 0.038†	0.515 ± 0.048	0.511 ± 0.015
AMP (μmol/g)	0.080 ± 0.016	0.208 ± 0.028*	0.068 ± 0.008†	0.119 ± 0.022†	0.082 ± 0.017†
ECP	0.899 ± 0.006	0.839 ± 0.014*	0.905 ± 0.007†	0.880 ± 0.013†	0.903 ± 0.006†
Creatine (μmol/g)	7.89 ± 0.29	8.16 ± 0.25	7.13 ± 0.22†	7.45 ± 0.31	7.61 ± 0.15
Glucose (μmol/g)	1.70 ± 0.06	1.47 ± 0.07*	2.23 ± 0.14†	1.86 ± 0.10	2.13 ± 0.12†
Pyruvate (μmol/g)	0.125 ± 0.008	0.132 ± 0.012	0.093 ± 0.013	0.116 ± 0.018	0.095 ± 0.010
Lactate (μmol/g)	2.20 ± 0.15	4.31 ± 0.16*	2.76 ± 0.15†	3.18 ± 0.17†	3.04 ± 0.27†
Lactate/pyruvate	17.8 ± 1.4	33.7 ± 2.7*	32.7 ± 3.3	31.7 ± 4.8	33.5 ± 3.7

Results are means ± SEM from six animals. Controls (group I) were injected with saline solutions only. Animals in other groups were injected with saline solution containing no carnitine (group II), 20 mmol L-carnitine/kg (group III), 20 mmol D-carnitine/kg (group IV) or 20 mmol acetyl-L-carnitine/kg (group V), and 30 min later with 15 mmol ammonium acetate/kg. The animals were immersed in liquid nitrogen 10 min after the injection of the second saline or ammonium acetate.

* $P < 0.05$ when compared with group I by Student's *t*-test.

† $P < 0.05$ when compared with group II by analysis of variance with Dunnett's test.

‡ $P < 0.05$ when compared with group III by analysis of variance with Tukey's test.

§ $P < 0.05$ when compared with group IV by analysis of variance with Tukey's test.

(groups III, IV and V), the alterations of brain energy metabolism were suppressed. In L-carnitine-treated animals (group III), the levels of ammonia, ADP, AMP, creatine and lactate were lower ($P < 0.05$), and PCr, ECP and glucose were higher than those in group II ($P < 0.05$). In D-carnitine-treated animals (group IV), the levels of ammonia, AMP and lactate were lower ($P < 0.05$), and PCr and ECP were higher than those in group II ($P < 0.05$). In acetyl-L-carnitine-treated animals (group V), the levels of ammonia, AMP and lactate were lower ($P < 0.05$), and PCr, ATP, ECP and glucose were higher than those in group II ($P < 0.05$). When compared among carnitine-treated animals (groups III, IV and V), the levels of ammonia were higher in group V than in group III ($P < 0.05$), PCr was higher in group V than in group IV ($P < 0.05$), and ATP was higher in group V than in group III or IV ($P < 0.05$). The level of pyruvate did not differ among the five groups.

Effects on levels of ammonia and urea in the blood. Administration of ammonium acetate caused a marked elevation of ammonia and urea concentrations in group II when compared with group I ($P < 0.005$) (Table 2). However, ammonia concentrations in animals treated with carnitine (groups III, IV and V) were lower than in untreated animals (group II) ($P < 0.05$). When compared among carnitine-treated groups, the level of ammonia in acetyl-L-carnitine-treated animals (group V) was higher than in L-carnitine-treated animals (group III) ($P < 0.05$). The levels of urea in carnitine-treated animals (groups III, IV and V) did not differ from that in untreated animals (group II).

DISCUSSION

L-Carnitine is widely present among tissues [18]. In humans, it is synthesized in liver, kidney and brain [19]. It has been well established that L-carnitine is essential for activated long-chain fatty acids to be carried across the inner mitochondrial membrane [18].

On the other hand, it has been shown that L-carnitine treatment suppresses octanoic acid-induced alterations of the staining of cytochrome oxidase (of mitochondria) in the choroid plexus in the rat brain [20]. We also found that L-carnitine suppresses changes of energy metabolites in the mouse brain under severe ischemia [21]. These observations suggest that carnitine may have a potential to protect the brain from various forms of insults that interfere with the energy supply.

In this study, we found that a single administration of ammonium acetate (15 mmol/kg) caused clear dysfunctions of the brain, i.e. the development of seizures and the alterations of brain energy metabolites, and treatment with L-carnitine delayed the onset of the seizures, reduced the number of seizures, preserved the brain energy metabolites, and lowered the ammonia concentration in the brain. These protective effects of L-carnitine against the neurotoxicity of ammonia agree well with our previous findings [11, 12] and those of other investigators. Hearn *et al.* [6] also reported a delay of the onset of the seizures and O'Connor *et al.* [3, 4] reported a decrease of ammonia concentration in the brain. In addition, we found that D-carnitine and acetyl-L-carnitine also suppressed the neurotoxicity of ammonia as L-carnitine did. Because all of the carnitine compounds examined lowered ammonia levels not only in the brain but also in the blood, these protective effects seem systemic.

The major system to remove ammonia in the body is the hepatic urea cycle [1]. It has been reported that L-carnitine increases urea synthesis in mice

Table 2. Effects of L-carnitine, D-carnitine and acetyl-L-carnitine on blood ammonia and urea concentrations in mice with hyperammonemia

	Control (group I)	Saline (group II)	L-Carnitine (group III)	D-Carnitine (group IV)	Acetyl-L-carnitine (group V)
Ammonia (μmol/mL)	0.07 ± 0.01 (8)	4.03 ± 0.28* (7)	2.05 ± 0.10† (8)	2.71 ± 0.22† (7)	2.84 ± 0.24†‡ (8)
Urea (μmol/mL)	8.08 ± 0.63 (6)	12.22 ± 0.88* (7)	12.00 ± 0.89 (6)	12.75 ± 0.81 (6)	11.32 ± 0.63 (8)

Results are means ± SEM. The number of samples in each group is given in parentheses. Controls (group I) were injected with saline solutions only. Animals in other groups were injected with saline solution containing no carnitine (group II), 20 mmol L-carnitine/kg (group III), 20 mmol D-carnitine/kg (group IV) or 20 mmol acetyl-L-carnitine/kg (group V), and 30 min later with 15 mmol ammonium acetate/kg. The animals were immersed in liquid nitrogen 10 min after the injection of the second saline or ammonium acetate.

* $P < 0.005$ when compared with group I by Student's *t*-test or Welch's *t*-test.
† $P < 0.05$ when compared with group II by analysis of variance with Dunnett's test.
‡ $P < 0.05$ when compared with group III by analysis of variance with Tukey's test.

given ammonium acetate, and this was considered to be a possible mechanism for the suppression of the neurotoxicity of ammonia by L-carnitine [22, 23]. On the other hand, L-carnitine has been reported to protect an ammoniotelic species (juvenile chinook salmon) from acute ammonia toxicity [8]. This indicates, at least in a certain kind of fish, that the urea cycle does not play a role in the carnitine-mediated protection against hyperammonemia. In our study, we saw no significant effects of carnitine on the concentration of urea in the blood. This might be because hyperammonemia induced in the present study was so high that it maximized the turnover of the urea cycle even in the untreated animals and carnitine could not further increase the urea production. If this assumption is correct, acceleration of the urea cycle may not be involved in the suppression of the toxicity of ammonia by carnitine.

Dilution of ammonia in the peritoneal cavity by carnitine solution was suggested in previous studies [10] as a mechanism for the beneficial effects of carnitine in hyperammonemia. The possibility of a simple dilution of ammonia in the peritoneal cavity was excluded in the present experiments because untreated animals were injected with the same amount of saline. Nevertheless, osmotic dilution or interference with the uptake of ammonia in the peritoneal cavity could not be ruled out. However, in the study by Bobyleva-Guarriero *et al.* [24], L-carnitine showed significantly protective effects against ammonia-induced mitochondrial dysfunction even when compared with 0.6 M NaCl solution. O'Connor *et al.* [25] also showed that L-carnitine is effective when injected intramuscularly or subcutaneously. These results are against the "osmoprotective" hypothesis concerning the beneficial effects of carnitine in hyperammonemia that were suggested previously [5]. In our study, the concentration of ammonia in the blood increased more than 29 times the normal level even when the animals were treated with carnitine, and carnitine did protect the brain. Hence, it appears that dilution of ammonia or interference with the absorption of ammonia in the peritoneal cavity does not play a major role in the protection of animals from hyperammonemia.

Recently, Ohtsuka and Griffith [7] reported that DL-aminocarnitine, acetyl-DL-aminocarnitine and palmitoyl-DL-aminocarnitine, potent inhibitors of carnitine acyltransferase, increase the mortality caused by ammonia, and they stressed the importance of L-carnitine-dependent metabolism for the protective effects against ammonia toxicity. However, we found that D-carnitine, which has been believed to be biologically inactive, suppressed ammonia-induced seizures and alterations of the brain energy metabolites as L-carnitine did, although the levels of ADP and creatine were lowered significantly only by L-carnitine. (The levels of these metabolites were not different between L- and D-carnitine-treated animals when the two groups were compared directly.) Moreover, since D-carnitine has been shown to have competitive inhibitory effects on carnitine acetyltransferase [26], the protective effects of carnitine seen in the present study cannot be explained solely by the "classical" function of

carnitine such as the transport of fatty acids across the inner mitochondrial membrane or the modulation of the acetyl-CoA/CoA ratio [18]. In an *in vitro* study by Bellei *et al.* [27], L-carnitine suppressed the mitochondrial swelling and the decrease of the respiratory rate caused by ammonia without fatty acids in the incubation mixture. This also suggests that the transport of fatty acids is not the mechanism of carnitine-mediated protection against ammonia toxicity.

Administration of acetyl-L-carnitine is known to supplement the acetyl groups and it also causes cholinergic effects in the brain in addition to the functions due to the carnitine moiety [28]. In the present study, when compared with L-carnitine, acetyl-L-carnitine lowered ammonia concentrations in the blood and brain less markedly; however, it better preserved the ATP level in the brains of mice given ammonium acetate. This suggests that the protection of the brain by acetyl-L-carnitine from hyperammonemia may involve mechanisms different from those of L- or D-carnitine. Although disputed, hyperammonemia has been reported to decrease the acetylcholine content in the brain [1], and recent studies revealed that choline content is lowered in patients with chronic hepatic encephalopathy [29, 30]. Hence, a part of the effects of acetyl-L-carnitine seen in the present experiments may be related to its actions on the cholinergic system in the brain [31].

In summary, our findings clearly confirmed the protective effects of carnitine against the neurotoxicity of ammonia, and suggested that the "classical" function of carnitine is not the sole mechanism since its analogues, including the D form, were similarly effective.

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