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EFFECT OF L-CARNITINE ON KETONE BODIES, REDOX STATE AND FREE AMINO ACIDS IN THE LIVER OF HYPERAMMONEMIC MICE

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Abstract—L-Carnitine stimulates urea synthesis in mice given a LD_{100} of ammonium acetate. Unprotected mice show decreased levels of hepatic ketone bodies and lowered NADH/NAD^+ ratio in both cytosol and mitochondria. L-Carnitine enhances markedly the production of β-hydroxybutyrate and raises the NADH/NAD^+ ratio in mitochondria. The alterations induced by ammonium acetate in the free aminoacid pool are prevented by L-carnitine. The results shown in this paper indicate that L-carnitine stimulates fatty acid oxidation as well as flux through the Krebs cycle in hyperammonemic mice and that these effects may be responsible for the increase in urea synthesis in these animals.

Severe liver disease and inherited hyperammonemic syndromes may lead to fatal dysfunction of the central nervous system because of the neurotoxicity of increased ammonia levels [1, 2]. The treatment of the neurological alterations which accompany hyperammonemia is largely empirical and not entirely satisfactory [3].

L-Carnitine administration prevents the lethal effects of acute hyperammonemia in mice [4–6]. This protective effect is accompanied by a marked reduction in blood and brain ammonia. Also, L-carnitine stimulates urea production following administration of high quantities of ammonia, although the effect is not immediate. Maximal urea levels in blood are seen about 1 hr after an LD_{100} of ammonium acetate is given to mice which had been injected previously with L-carnitine [4]. No changes in ATP [7] nor in *N*-acetylglutamate [6] over those induced by ammonia alone were observed in the liver of the protected mice.

Oleate or β-hydroxybutyrate stimulate urea synthesis from ammonia in isolated hepatocytes. It has been suggested that this effect is related to the redox state of liver mitochondria [8]. Since L-carnitine is essential for the complete oxidation of long-chain fatty acids in mitochondria [9], the enhancing effect of L-carnitine on urea synthesis might depend on stimulation of fatty acid oxidation. Therefore we have determined the hepatic levels of some metabolites related to fatty acid oxidation, redox state and glucose and amino acid metabolism.

In this paper we show that enhanced fatty acid oxidation appears responsible for the stimulation of urea synthesis in the liver of ammonia- and L-carnitine-treated mice.

MATERIALS AND METHODS

Male Swiss albino mice weighing 25–30 g, fed a standard diet *ad libitum* were used. Hyperammonemia was induced by a single lethal intraperitoneal injection of 12 mmol ammonium acetate/kg body weight (as a 0.8 M solution). Clear symptoms of ammonia toxicity appeared rapidly. The mice became hyperexcitable soon followed by drowsiness. Noise-induced seizures preceded coma, with frequent clonic convulsions. Death occurred in all cases, generally after a sustained tonic convulsion, 10–15 min after the injection of ammonium acetate.

L-Carnitine was injected intraperitoneally 30 min before injecting ammonium acetate. The carnitine-protected mice did not exhibit symptoms of ammonia toxicity. The dose of L-carnitine administered (16 mmol/kg body weight) is the lowest dose affording total protection against an LD_{100} of ammonium acetate [4].

Blood urea was measured in samples from the tail vein at intervals after the injection of ammonium acetate by the colorimetric method of Hunninghake and Grisolía [10]. Ten or 60 min after the administration of ammonium acetate, the mice were killed, their livers removed and frozen in liquid nitrogen.

The following metabolites were determined enzymatically in liver homogenates: acetoacetate [11], β-hydroxybutyrate [12], lactate [13] and pyruvate [14].

Amino acids in liver homogenates were measured by high-performance liquid chromatograph (Pye Unicam). Sample preparation and technical conditions were as described elsewhere [15].

L-Carnitine hydrochloride (Sigma-Tau, Italy), was a gift from Laboratorios Glaxo, Spain.

RESULTS

As seen in Fig. 1, blood urea rose in the animals given ammonium acetate. In the first minutes the increase was similar in both protected and unprotected mice. The unprotected animals die in *ca* 15 min. Blood urea continued to rise in mice previously given L-carnitine, reaching a maximal plateau 1 hr after the ammonium acetate injection. Metab-

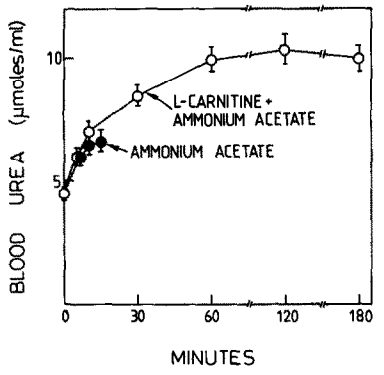

Fig. 1. Effect of L-carnitine on urea levels in blood of mice injected with a lethal dose of ammonium acetate. The animals received 16 mmoles L-carnitine/kg body weight intraperitoneally, 30 min before receiving 12 mmoles ammonium acetate by also i.p. Urea was measured in blood samples from the tail vein, as indicated in Materials and Methods. The results are the mean ± SD of four mice.

olites were therefore measured 10 and 60 min after ammonium acetate administration.

Table 1 shows the effects of ammonium acetate and L-carnitine on the levels of ketone bodies in the liver. At the time of death, the unprotected mice had decreased levels of both acetoacetate and β-hydroxybutyrate. The intramitochondrial NADH/NAD$^+$ ratio, calculated from the quotient β-hydroxybutyrate/acetoacetate [16], was lower in the animals injected with ammonium acetate. L-Carnitine alone increased markedly β-hydroxybutyrate, and decreased acetoacetate slightly, which increased the NADH/NAD$^+$ ratio in mitochondria. L-Carnitine, when given prior to ammonium acetate, prevented the ammonia-induced decrease in ketone bodies and thus in the NADH/NAD$^+$ ratio, but this effect was delayed. While at 10 min after ammonium acetate, L-carnitine had only a slight effect, after 60 min β-hydroxybutyrate was increased by 185% and the NADH/NAD$^+$ ratio by 360%.

The cytosolic redox state can be calculated from the ratio lactate/pyruvate, as an indicator of the NADH/NAD$^+$ quotient [16]. As seen in Table 2, ammonium acetate decreased this ratio since lactate was decreased slightly. L-Carnitine alone should not significantly affect the cytosolic NADH/NAD$^+$, although it did decrease slightly both lactate and pyruvate. Contrary to the effect on mitochondrial redox state, L-carnitine administration prior to ammonium acetate enhanced the ammonia-induced shift in NADH/NAD$^+$ towards a more oxidized state, resulting from a further decrease in lactate.

In Fig. 2 are shown the levels of free amino acids related to urea synthesis in the liver of mice given ammonium acetate and/or L-carnitine. No significant variations in the amino acids involved in the urea cycle (i.e. citrulline, ornithine and arginine) were noted as a result of ammonium acetate or of L-carnitine. However, there was a marked increase in ornithine in L-carnitine-protected mice 60 min after the administration of ammonium acetate. The most striking variations were observed in alanine, aspartate and glutamate, which were markedly increased by ammonium acetate and partially reduced when L-carnitine was given previously. Asparagine and glutamine, which were not affected by ammonium acetate alone, were decreased by L-carnitine.

Regarding the amino acids not closely related to urea synthesis (Fig. 3), taurine, histidine, lysine, tyrosine, methionine and tryptophan were increased by ammonia. Previous treatment with L-carnitine resulted in normal levels of these amino acids at 60 min after injection of ammonium acetate, except for lysine which was markedly increased. Serine, which was slightly reduced by ammonium acetate, showed a further decrease in L-carnitine treated animals.

The taurine/glycine ratio, which has been considered as an indicator of metabolic stress [17], is markedly increased in ammonia-intoxicated mice. L-Carnitine partially prevents this increase by stabilizing the levels of taurine.

The ratio of branched-chain amino acids (valine,

Table 1. Effect of ammonium acetate and L-carnitine on levels of acetoacetate and β-hydroxybutyrate in the liver of mice

Treatment	Acetoacetate (μmoles/g)	β-Hydroxybutyrate (μmoles/g)	NADH/NAD$^+$ (×10^9)
None	0.057 ± 0.001	0.139 ± 0.003	3.46
Ammonium acetate, 10 min	0.035 ± 0.002	0.063 ± 0.002	2.56
L-Carnitine + ammonium acetate, 10 min	0.042 ± 0.008	0.079 ± 0.009	2.81
L-Carnitine + ammonium acetate, 60 min	0.039 ± 0.003	0.282 ± 0.003	10.27
L-Carnitine, 40 min	0.039 ± 0.001	0.230 ± 0.060	8.38
L-Carnitine, 90 min	0.040 ± 0.001	0.275 ± 0.007	9.75

NADH/NAD$^+$ mitochondria = $K \times$ (β-hydroxybutyrate)/(acetoacetate): $K = 1.49 \times 10^{-9}$.

Mice were injected intraperitoneally with 16 mmoles L-carnitine/kg body weight, 30 min before receiving 12 mmoles ammonium acetate by the same route. The mice were sacrificed 10 min or 60 min after injection of the ammonium acetate, together with the controls. Acetoacetate and β-hydroxybutyrate were determined in the livers as indicated in Materials and Methods. The results are the mean ± SD of four mice.

Table 2. Effect of ammonium acetate and L-carnitine on levels of lactate and pyruvate in the liver of mice

Treatment	Pyruvate (μmoles/g)	Lactate (μmoles/g)	NADH/NAD$^+$ ($\times 10^2$)
None	0.14 ± 0.02	0.99 ± 0.29	20.50
Ammonium acetate, 10 min	0.12 ± 0.03	0.77 ± 0.07	18.62
L-Carnitine + ammonium acetate, 10 min	0.12 ± 0.01	0.46 ± 0.15	11.11
L-Carnitine + ammonium acetate, 60 min	0.14 ± 0.01	0.37 ± 0.12	7.62
L-Carnitine, 40 min	0.11 ± 0.02	0.85 ± 0.10	22.39
L-Carnitine, 60 min	0.10 ± 0.01	0.62 ± 0.09	17.98

NADH/NAD$^+$ cytosol = $K \times$ (lactate)/(pyruvate): $K = 2.9 \times 10^{-2}$ M

The animals received 16 mmoles L-carnitine/kg body weight intraperitoneally, 30 min before receiving 12 mmoles ammonium acetate/kg body weight by the same route. The mice were sacrificed 10 min or 60 min after the ammonium acetate, i.e. 40 min or 90 min after the L-carnitine. Lactate and pyruvate were determined in liver as indicated in Materials and Methods. The results are the mean ± SD of four mice.

leucine and isoleucine) to aromatic amino acids (phenylalanine and tyrosine) is decreased in conditions of fulminant hepatic failure [18, 19]. As can be seen, ammonium acetate decreased markedly this ratio, while it was restored by the prior injection of L-carnitine, 60 min after the administration of ammonium acetate.

DISCUSSION

The results presented here show a concomitant stimulation of urea synthesis by L-carnitine in animals given an LD_{100} of ammonium acetate with an enhancement of fatty acid oxidation in liver. L-Carnitine, by increasing β-hydroxybutyrate and thus the NADH/NAD$^+$ ratio in mitochondria, is able to correct the changes induced by ammonia injection (Table 1). That the increasing effect of L-carnitine on the NADH/NAD$^+$ ratio must result from intramitochondrial generation of reducing equivalents is supported by the lack of variation of this ratio in the cytosol (Table 2).

Meijer *et al.* [8] demonstrated that the addition of oleate or β-hydroxybutyrate to hepatocytes incubated with ammonium chloride stimulated markedly the synthesis of urea, while the addition of acetoacetate had an inhibitory effect. They concluded that the stimulatory effect of oleate and β-hydroxybutyrate on urea synthesis resulted from both an increase in reducing equivalents and enhanced ATP generation within the mitochondria. Since the main physiological role of L-carnitine is to facilitate the entry of fatty acids into the mitochondria [8], the stimulation of urea synthesis by L-carnitine agrees well with the postulation of Meijer *et al.*

The stoichiometry of the urea cycle requires that one mole of ammonia is converted to carbamyl phosphate in the mitochondria while in the presence of high ammonia concentration a second mole of ammonia will form glutamate via glutamate dehydrogenase. Transamination of glutamate will form aspartate which should then leave the mitochondria and condense with citrulline yielding argininosuccinate. The accumulation of aspartate and alanine observed in mice given ammonium acetate indicates an overcompensation via transamination for the

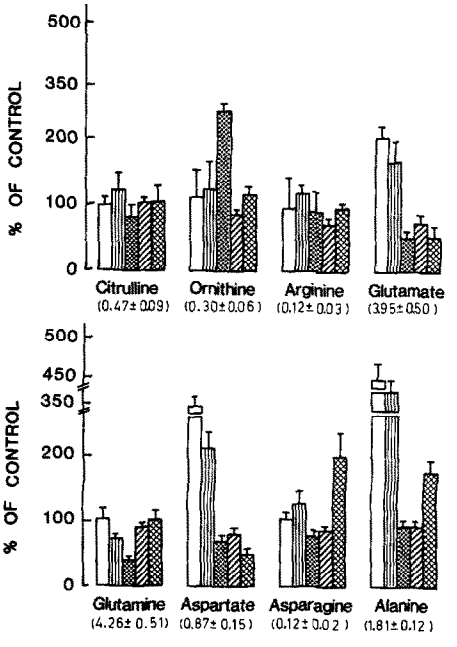

Fig. 2. Effect of L-carnitine on the levels of amino acids closely related to urea synthesis in the liver of mice injected with a lethal dose of ammonium acetate. The animals received 16 mmoles L-carnitine/kg body weight i.p., 30 min before receiving 12 mmoles ammonium acetate/kg body weight by the same route. The mice were sacrificed 10 min or 60 min after the ammonium acetate, i.e. 40 min or 90 min after L-carnitine. Amino acids were determined as indicated in Material and Methods. Results are the mean ± SD of 4–8 mice and are represented as percent of controls which are taken as 100%. The control values for each amino acid appear at the bottom of each histogram. □, ammonium acetate, 10 min; ▥, L-carnitine + ammonium acetate, 10 min; ▩, L-carnitine + ammonium acetate, 60 min; ▨, L-carnitine, 40 min; ▩, L-carnitine, 90 min.

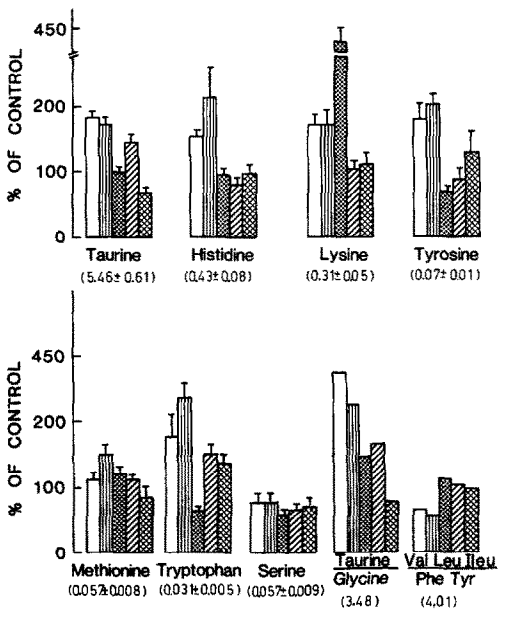

Fig. 3. Effect of L-carnitine on the levels of amino acids not closely related to urea synthesis in the liver of mice injected with a lethal dose of ammonium acetate. The animals received 16 mmoles L-carnitine/kg body weight i.p., 30 min before receiving 12 mmoles ammonium acetate/kg body weight by the same route. The mice were sacrificed 10 min or 60 min after the ammonium acetate, i.e. 40 min or 90 min after L-carnitine. Amino acids were determined as indicated in Material and Methods. Results are the mean ± SD of 4–8 mice and are represented as per cent of the controls which are taken as 100%. The control values for each amino acid appear at the bottom of each histogram. Although not shown, no significant variations were observed in glycine, leucine, valine, threonine and isoleucine: □, ammonium acetate, 10 min; ▥, L-carnitine + ammonium acetate, 10 min; ▩, L-carnitine + ammonium acetate, 60 min; ▨, L-carnitine, 40 min; ▩, L-carnitine, 90 min.

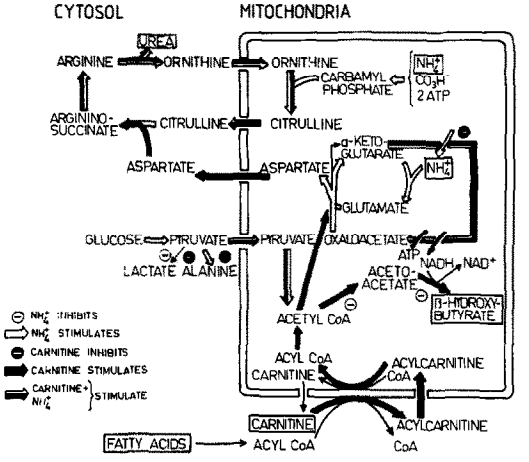

Fig. 4. Metabolic scheme showing the possible points for the stimulation of urea synthesis by L-carnitine.

operation of the urea cycle. The previous administration of L-carnitine reduces the magnitude of the accumulation of glutamate, aspartate and alanine. Since aspartate is used for the synthesis of argininosuccinate, the increase in urea synthesis induced by L-carnitine could also be due to an effect on this step via increase of ATP. It is interesting that argininosuccinate synthetase may become rate-limiting for urea synthesis in conditions of an excess of ammonia [20, 21].

Ammonia stimulates glycolysis in liver [22, 23] and increases the flux through pyruvate dehydrogenase [23, 24], thereby decreasing lactate (refs. 25 and 26 and Table 2). L-Carnitine stimulates pyruvate and α-ketoglutarate dehydrogenases [26]. It is likely that these may explain the further decrease in lactate and the reduced accumulation of alanine seen in mice given L-carnitine prior to ammonium acetate, suggesting a further enhancement of pyruvate utilization via the Krebs cycle. By this additional effect, L-carnitine might ameliorate the linkage between increased glycolysis and ATP production in ammonia-intoxicated mice. A metabolic scheme of the possible mechanism for the stimulation of urea synthesis by L-carnitine is shown in Fig. 4.

The levels of arginine and citrulline were not affected by L-carnitine (Fig. 3), although there was a remarkable increase of unknown mechanism, in ornithine when ammonia was also given.

In addition to the effects of L-carnitine on energy metabolism and urea synthesis in liver, some of the alterations induced by acute ammonia intoxication are ameliorated by L-carnitine. Thus, increases in taurine, tyrosine, methionine and tryptophan were prevented by L-carnitine. It is interesting that increased levels of methionine [26] and tryptophan [18] have been implicated as participating in the pathogenesis of hepatic encephalopathy. Also, the ratio of branched-chain amino acids (valine + leucine + isoleucine) to aromatic amino acids (phenylalanine + tyrosine) was normalized after administration of L-carnitine. This ratio is commonly decreased in patients with severe liver disease, a condition in which hyperammonemia is often seen [18, 19].

The ratio taurine/glycine has been proposed as an indicator of metabolic stress, although its physiological meaning is unknown [17]. Certainly, this ratio increases very rapidly in fish [17] and invertebrates [27] exposed to high concentrations of ammonia. As seen in Fig. 3, in the ammonia-intoxicated mice, the taurine/glycine ratio was increased, while it was normalized when L-carnitine was administered.

The results presented in this paper show that L-carnitine stimulates *in vivo* fatty acid oxidation and that this effect is responsible for the increase in urea synthesis seen in mice given L-carnitine prior to an LD_{100} of ammonium acetate. The increase in acetylCoA and intramitochondrial reducing equivalents following β-oxidation, as well as the stimulation of flux through the Krebs cycle by L-carnitine appear to be involved in the metabolic changes induced in the liver of these mice. Also, L-carnitine seems to abolish some of the alterations in the amino acid pool induced by ammonia on the liver.

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