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Nitroarginine, an Inhibitor of Nitric Oxide Synthetase, Attenuates Ammonia Toxicity and Ammonia-Induced Alterations in Brain Metabolism

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We have proposed that acute ammonia toxicity is mediated by activation of the N-methyl-D-aspartate type of glutamate receptors. MK-801, a selective antagonist of these receptors, prevents death of animals induced by acute ammonia intoxication as well as ammonia-induced depletion of ATP. It seems therefore that, following activation of the N-methyl-D-aspartate receptors, the subsequent events in ammonia toxicity should be similar to those involved in glutamate neurotoxicity. As it has been shown that inhibitors of nitric oxide synthetase such as nitroarginine prevent glutamate toxicity, we have tested whether nitroarginine prevents ammonia toxicity and ammonia-induced alterations in brain energy and ammonia metabolites. It is shown that nitroarginine prevents partially (≈ 50%), but significantly death of mice induced by acute ammonia intoxication. Nitroarginine also prevents partially ammonia-induced depletion of brain ATP. It also prevents completely the rise in glucose and pyruvate and partially that in lactate. Injection of nitroarginine alone, in the absence of ammonia, induces a remarkable accumulation of glutamine and a decrease in glutamate. The results reported indicate that nitroarginine attenuates acute ammonia toxicity and ammonia-induced alterations in brain energy metabolites. The effects of MK-801 and of nitroarginine are different, suggesting that ammonia can induce nitric oxide synthetase by mechanisms other than activation of N-methyl-D-aspartate receptors.

KEY WORDS: Ammonia; nitric oxide; nitroarginine; NMDA receptor; neurotoxicity; brain metabolism.

INTRODUCTION

Hyperammonemia is considered one of the main factors that mediates hepatic encephalopathy. It is also well known that injection into animals of large doses of ammonia leads to death of animals. However, in spite of much work, the molecular mechanism(s) of ammonia toxicity and the role of ammonia in the pathogenesis of hepatic encephalopathy remain unclear. Several hypotheses have been proposed to explain these processes (1–6); however, none of these hypotheses is completely consistent with all the experimental observations.

Acute administration into normal animals of large toxic doses of ammonia is associated with marked alterations in brain metabolites, including increased lactate, pyruvate, glucose, and mitochondrial [NAD^+]/[NADH] and decreased glycogen, cytosolic [NAD^+]/[NADH], and, at a later step, decreased ATP content (7–10). Ammonia intoxication also results in a large increase in the brain content of glutamine (7,10). It has been suggested

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that changes in brain energy metabolites and depletion of ATP would play an important role in the origin of ammonia-induced coma and death.

We have shown that MK-801, a selective antagonist of the NMDA type of glutamate receptors, prevents death of mice and rats induced by acute ammonia intoxication (6). We proposed that acute ammonia toxicity is mediated by activation of these receptors. MK-801 also prevents some of the effects induced by acute ammonia intoxication in brain such as proteolysis of microtubule-associated protein MAP-2 (11,12) and depletion of ATP (13). These results suggest that activation of the NMDA receptor is responsible for ammonia-induced depletion of ATP and for death of animals. It seems therefore likely that, following activation of this receptor, the molecular mechanism of ammonia toxicity would be similar to that of glutamate neurotoxicity. Sustained activation of glutamate receptors is neurotoxic, leading to the death of most neurons. The molecular mechanism of glutamate neurotoxicity is now being extensively studied. In many systems, glutamate toxicity is nearly completely prevented by MK-801. This suggests that glutamate neurotoxicity is mainly mediated by activation of the NMDA receptor. Activation of this receptor leads to the opening of the associated ion channel, allowing the entry of Ca^{2+} and Na^{+} into the neuron and leading to activation of nitric oxide synthetase (14–16). The opening of the ion channel is an essential step in the mediation of glutamate toxicity. However, the subsequent mechanism leading to neuronal death remains unclear (17–19). It has been shown that inhibitors of nitric oxide synthetase (NOS) prevent glutamate toxicity (20–22), suggesting a role for this enzyme in the mediation of glutamate toxicity. We therefore considered of interest to study if NOS is involved in the mechanism of ammonia toxicity. We tested whether nitroarginine, a selective inhibitor of NOS, is able to prevent acute ammonia toxicity in animals and/or ammonia-induced alterations in energy and ammonia metabolites in rat brain.

EXPERIMENTAL PROCEDURE

For experiments on death of animals caused by acute ammonia intoxication male Swiss albino mice weighing 25–30 *g* were used. For experiments on ammonia-induced alterations of brain metabolites male Wistar rats weighing 230–270 *g* were used. Four groups of 8 rats were used (two different experiments with 4 rats per group for each experiment). One group was injected intraperitoneally with 7 mmol/Kg of ammonium acetate and killed 15 min later. This time was chosen on the bases of previous experiments in which it was found that the effects of acute ammonium intoxication on brain metabolites can be clearly seen 15 min after injection (10). To assess whether nitric oxide synthetase plays a role in the ammonia-induced alteration in brain metablites, another group was injected i.p. with 45 mg/Kg of nitroarginine (a selective inhibitor of NOS) 10 min before injecting ammonium acetate. These conditions were chosen because they provide the highest protection against ammonia-induced death of animals (see Table I). Similar results were obtained whether nitroarginine was injected 10 or 15 min before ammonium acetate. Another group was injected only with nitroarginine and killed 25 min later. The fourht group consisted of the controls. These animals were not injected. In previous experiments we injected the controls with saline (10), but we have found that, under the conditions used, injection of saline do not affect the content in brain of the metabolites studied in this work.

Rats were killed by decapitation and brain was immediately removed and freeze-clamped. The frozen tissue was powdered under liquid nitrogen in a mortar and brain extracts were prepared with perchloric acid/ethanol at −10°C. Metabolites were determined as previously described (13). Briefly, glucose was determined enzymatically as described by Kunst et al (23); glycogen was hydrolyzed with sulfuric acid and then determined as glucose. ATP, ADP and AMP were determined enzymatically as described by Trantschold et al (24) and by Jaworet and Welsch (25). Lactate, pyruvate, glutamate and glutamine were determined enzymatically as described by Noll (26), Lamprecht and Heinz (27), Bernt and Bergmeyer (28) and Lund (29), respectively. Inorganic phosphorous was determined as indicated by Taussky and Shorr (30) and ammonia as described by Bergmeyer and Beutler (31). The phosphorylation potential was calculated according to Atkinson and Walton (32). Glutamine synthetase activity was assayed as described by Meister (33). Statistical analysis of data was carried out using Student's t test.

RESULTS

As shown in Table I, nitroarginine is able to afford a partial but significant protection against ammonia-induced death of mice. When mice were injected with a dose of 30 mg/Kg, i.p., there was a partial, non significant protection. The protective effect was significant and maximal (≈ 50% survivors) at the dose of 45 mg/Kg, i.p.. Larger doses (e.g. 60 mg/Kg) did not afford further protection. Nitroarginine methylester, another inhibitor of NOS, also prevents partially the toxicity of ammonia.

The results of the pretreatment with nitroarginine on the alteration of brain metabolites induced by acute ammonium administration are shown in Table II. Injection of 7 mmol/Kg of ammonium acetate leads to a remarkable decrease (51%) of brain ATP. Injection of nitroarginine alone also reduces slightly (17%) ATP content in brain. Ammonia-induced depletion of ATP is partially prevented in rats injected previously with nitroarginine; in these rats ATP content is slightly lower (88%) than in rats injected with nitroarginine alone. Nitroarginine also prevents partially the reduction in the phosphorylation potential induced by ammonium injection (Table II).

Ammonium injection decreases glycogen by 55% and increases glucose by 123%. Nitroarginine completely prevented the ammonia-induced rise of glucose

Table I. Effect of Inhibitors of Nitric Oxide Synthetase on Ammonia Toxicity in Mice

	Animals died/animals injected				Animals died
Pretreatment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean ± SD (% of injected)
None	10/10	9/11	11/12	11/11	93.5 ± 7.4
Nitroarginine 30 mg/Kg	—	8/10	4/11	9/9	72.0 ± 26.7
Nitroarginine 45 mg/Kg	5/10	6/11	6/12	5/9	52.5 ± 2.6*
NAME 75 mg/Kg	8/10	—	6/12	8/12	68.3 ± 13.1

Groups of mice were injected intraperitoneally with 12 mmol/Kg of ammonium acetate. Nitroarginine or NAME (nitroarginine methylester) were injected i.p. 10 min before ammonium injection. Values indicated by * are significantly different ($p \leq 0.001$) from those for animals injected only with ammonia.

Table II. Effect of Pretreatment with Nitroarginine on the Alteration of Brain Metabolites Induced by Ammonium Injection

	Compound Injected			
Metabolite	None	Ammonia	Nitroarg.	Nitroarg. + Ammonia
ATP	1.93 ± 0.04	0.94 ± 0.07[a]	1.60 ± 0.16[a]	1.40 ± 0.25[a,b]
ADP	0.68 ± 0.02	0.86 ± 0.04[a]	0.74 ± 0.06	0.70 ± 0.07[b]
AMP	0.05 ± 0.01	0.08 ± 0.01[a]	0.07 ± 0.01	0.06 ± 0.01[b]
Pi	1.20 ± 0.10	1.76 ± 0.18[a]	1.10 ± 0.10	1.28 ± 0.07[b]
Phosphorylation potential	2345 ± 225	676 ± 105[a]	2002 ± 327	1650 ± 275[a,b]
Glycogen	2.9 ± 0.1	1.3 ± 0.1[a]	2.8 ± 0.2	1.9 ± 0.3[a,b,c]
Glucose	0.81 ± 0.04	1.81 ± 0.15[a]	0.70 ± 0.07	0.75 ± 0.09[b]
Lactate	1.45 ± 0.03	3.02 ± 0.13[a]	1.31 ± 0.05	1.83 ± 0.07[a,b,c]
Pyruvate	88 ± 3	146 ± 13[a]	72 ± 8	81 ± 4[b]
α-ketoglutarate	83 ± 2	82 ± 6	87 ± 5	83 ± 8
Ammonia	0.34 ± 0.04	3.08 ± 0.03[a]	0.38 ± 0.03	2.35 ± 0.15[a,b,c]

Groups of 8 rats were used. One group (column labeled as Ammonia) was injected i.p. with 7 mmol/Kg of ammonium acetate and killed 15 min later. Another group (column labeled as Nitroarg. + Ammonia) was injected i.p. with 45 mg/Kg of nitroarginine, followed, 10 min later, by injection (i.p.) of 7 mmol/Kg of ammonium acetate. Rats were killed 15 min after ammonium injection. The group presented in the column labeled as Nitroarg. was injected i.p. with 45 mg/Kg of nitroarginine and killed 25 min later. The control group is presented in the column labeled as None. Brain metabolites were determined as described in Materials and Methods. Values are given as μmol/g except for pyruvate and α-ketoglutarate, which are given as nmol/g and for phosphorylation potential which is given as l/mol.

[a]significantly different ($p \leq 0.001$) from controls.

[b]significantly different ($p \leq 0.001$) from animals injected with ammonium acetate.

[c]significantly different ($p \leq 0.001$) from animals injected with nitroarginine.

and partially prevented the decrease in glycogen. Pyruvate and lactate increased by 66% and 108%, respectively, 15 min after ammonium injection. These changes were nearly completely prevented by prior injection of nitroarginine: pyruvate remained at control values while lactate increased only by 30% (Table II). The content of α-ketoglutarate was not affected by any of the treatments. Ammonia increased ≈ 9-fold in rats injected with ammonia and ≈ 7-fold in those injected with both nitroarginine and ammonia.

The effects of ammonium and nitroarginine injection on glutamine and glutamate are shown in Fig. 1. Ammonium injection induces a large increase (138%) in glutamine and a decrease (28%) in glutamate. Interestingly, injection of nitroarginine alone produces a similar change; glutamine increases by 162% while glutamate decreases by 33%. In rats injected with both nitroarginine and ammonium acetate, glutamine increased by 192% and glutamate decreased by 26%. It is therefore clear that nitroarginine did not prevent accumulation of

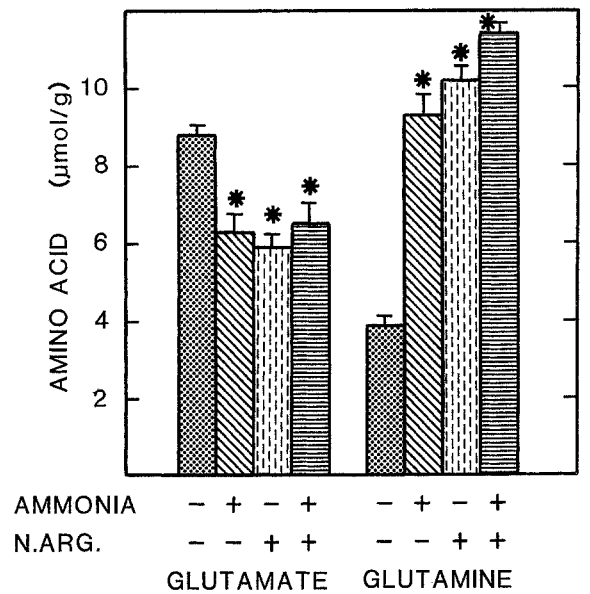

Fig. 1. Effect of pretreatment with nitroarginine on the ammonia-induced alterations of brain glutamate and glutamine. Groups of eight rats were injected i.p. with 7 mmol/Kg of ammonium acetate with or without previous injection of 45 mg/Kg of nitroarginine (i.p. 10 min before injecting ammonium acetate). Rats were killed 15 min after ammonium injection and glutamate and glutamine were determined as indicated in Experimental Procedure. Rats injected only with nitroarginine were killed 25 min after injection. Values are the mean ± SD of duplicate samples from eight rats. Values labeled with asterisk are significantly different ($p \leq 0.001$) from controls.

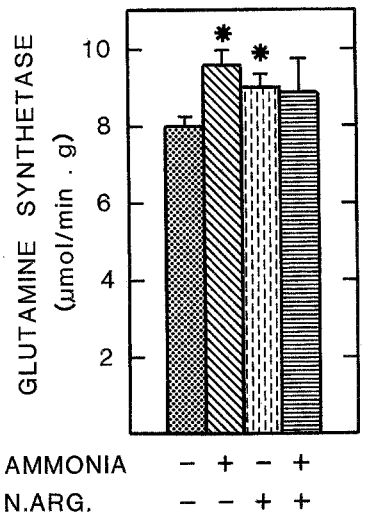

Fig. 2. Effect of ammonium and/or nitroarginine injection on glutamine synthetase activity in rat brain. Groups of eight rats were injected as in Fig. 1. Glutamine synthetase was determined as indicated in Experimental Procedure. Values are the mean ± standard deviation of duplicate samples from eight rats per group. Values labeled with asterisk are significantly different ($p \leq 0.001$) from controls.

glutamine induced by ammonia, but induces a similar accumulation by itself (Fig. 1).

We then tested the effects of ammonia and nitroarginine injection on glutamine synthetase. As shown in Fig. 2, the in vitro activity of glutamine synthetase is slightly increased in brains from rats injected with ammonia (18%) or with nitroarginine. The activity in rats injected with both compounds is not significantly different from controls.

DISCUSSION

Ammonia toxicity was first reported in the laboratory of Pavlov a century ago (34). The effects of ammonium injection on brain ammonia and energy metabolites have been extensively studied. However, the mechanism by which ammonia intoxication leads to depletion of brain ATP remains unclear. Bessman and Bessman (1) found that, in hyperammonemic patients, brain removes ammonia from blood. These authors suggested that incorporation of ammonia into glutamate and glutamine would deplete α-ketoglutarate, thus interfering with the Krebs cycle and leading to ATP depletion. However, it has been later shown that ammonia-induced ATP depletion is not associated with decreased content of α-ketoglutarate in the whole brain (9,10). Although depletion of α-ketoglutarate in certain areas of the brain or in some cellular types can not be ruled out, it seems that depletion of α-ketoglutarate would not explain depletion of ATP in whole brain.

It has been suggested that ATP depletion induced by acute ammonia intoxication may be a consequence of the large increase in glutamine which synthesis requires ATP consumption. We show here (Fig. 1), that injection of nitroarginine alone or followed by ammonium acetate induces a remarkable accumulation of glutamine, reaching levels higher than those induced by injection of ammonium acetate alone. However, this increase in glutamine is not associated with a large depletion of ATP, as is the case for ammonia-injected rats (Table II). Also, we have recently shown that MK-801 completely prevents ammonia-induced ATP depletion while does not reduces but enhances glutamine accumulation (13). These results indicate that ATP depletion is not a consequence of the increased content of glutamine.

Accumulation of glutamine in brain could be due to increased synthesis, decreased break-down by glutaminase or altered transport through the blood-brain barrier.

In acute ammonia intoxication it seems clear that increased synthesis to detoxify ammonia contributes significantly to accumulation of glutamine, although decreased activity of glutaminase in vivo can not be discarded. For rats injected with nitroarginine a significant contribution of altered break-down or transport of glutamine can not be ruled out.

It is well known that glutamate, when in excess, is neurotoxic. In many systems glutamate neurotoxicity is mediated by activation of the NMDA receptor (35,36). We propose that acute ammonia toxicity is mediated by activation of the NMDA receptor. In fact, MK-801, a selective antagonist of this receptor, completely prevents death of mice and rats injected with lethal doses of ammonium acetate (6). Moreover, MK-801 also completely prevents ATP depletion induced by injection of 7 mmol/Kg of ammonium acetate in rats (13). However, MK-801 did not prevent significantly ammonia-induced changes in glycogen, glucose, ketone bodies, glutamate or glutamine and prevents only partially changes in lactate and pyruvate (13). This indicates that the ammonia-induced effects are not exclusively coupled to the NMDA receptor-mediated effects but activation of this receptor is an essential step in the processes leading to ATP depletion and to death of animals.

As it has been proposed that activation of nitric oxide synthetase is an essential step in the mediation of glutamate neurotoxicity, we have tested here whether nitroarginine can prevent the toxic effects of ammonia.

Nitroarginine significantly prevents death of mice injected with 12 mmol/Kg of ammonium acetate; however, the protection is not complete (Table I). In contrast, MK-801 prevents completely death of mice and rats injected with large doses of ammonium acetate (6). We do not know whether injection of 45 mg/kg of nitroarginine inhibits completely NOS activity in vivo. This dose completely prevents ammonia-induced changes in glucose or pyruvate (Table II). Moreover, injection of larger doses did not afford additional protection against ammonia-induced death of mice. This suggests that the dose used is able to prevent most nitric oxide-mediated effects of acute ammonia intoxication. However, it can not be ruled out that incomplete protection could be due to incomplete inhibition of ammonia-induced activation of NOS. Incomplete protection could also be due to the fact that nitric oxide would not be the main factor responsible for neurotoxicity but only potentiates the effects mediated by another pathway (insensitive to nitroarginine) induced by activation of the NMDA receptor.

The effects of nitroarginine and of MK-801 on ammonia-induced alterations in brain metabolites are quite different. MK-801 completely prevents changes in ATP but not changes in glucose and only slightly those in lactate and pyruvate. In contrast, nitroarginine prevents completely the rise in glucose and pyruvate and nearly completely that of lactate while prevents only partially depletion of ATP. It should be noted that glutamine synthesis and glycogen hydrolysis occur mainly in astrocytes. On the other hand, it is considered that NMDA receptors are located mainly in neurons and are absent in normal astrocytes. Most of the metabolic changes induced by ammonia intoxication (e.g. altered glutamine synthesis and glycogen metabolism) appear to occur in astrocytes. It is therefore not surprising that MK-801, an antagonist of the NMDA receptor, would not be able to prevent ammonia-induced alterations in glycolysis or glutamine synthesis. MK-801 could prevent these effects if they were mediated by nitric oxide synthesized in neurons following activation of NMDA receptor. As NO is a diffusible second messenger, it could influence glycolysis in neighboring astrocytes. The fact that nitroarginine prevents ammonia-induced alterations in glycolysis suggests that nitric oxide (NO) could mediate this effect of acute ammonia intoxication. However, as these alterations are not prevented by MK-801, this suggests that the NO responsible for this effect is also produced after blocking the NMDA receptor. In other words, ammonia could induce the formation of NO by a pathway different of NMDA receptor activation. In fact, it has been reported that ammonia may generate nitric oxide in aorta and liver of the rat (37,38).

The fact that MK-801 prevents depletion of ATP induced by acute ammonia intoxication but not changes in glycolysis suggests that alterations in glycolysis are not responsible for ATP depletion. Moreover, as MK-801 acts on neurons, it seems likely that ATP depletion occur mainly in these cells. In contrast, changes in glycolysis occur mainly in astrocytes which contain most of the brain glycogen.

In summary, the above results, together with those previously described (6,11,13), suggest that acute ammonia intoxication leads to activation of the NMDA receptor which mediates brain ATP depletion and the processes leading to death of the animals. Acute ammonia administration would also induce NOS activity by a mechanism independent of NMDA receptor activation. The NO formed would mediate alterations in glycolysis and would potentiate the toxic effects induced by activation of NMDA receptor. Further experiments to clarify the target molecules modulated by ammonia-induced nitric oxide can help to elucidate the molecular events induced by ammonia in brain.

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