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Nitroarginine, an Inhibitor of Nitric Oxide Synthase, Prevents Changes in Superoxide Radical and Antioxidant Enzymes Induced by Ammonia Intoxication

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Injection of large doses of ammonium salts leads to the rapid death of animals. However, the molecular mechanisms involved in ammonia toxicity remain to be clarified. We reported that injecting ammonium acetate (7 mmol/kg) to rats increases the production of superoxide and reduces the activities of some antioxidant enzymes in rat liver and brain. We proposed that these effects induced by ammonia intoxication would be mediated by formation of nitric oxide. To test this possibility we tested whether injection of nitroarginine, an inhibitor of nitric oxide synthase, prevents the effects of ammonia intoxication on antioxidant enzymes and superoxide formation. Following injection of ammonia, glutathione peroxidase, superoxide dismutase and catalase activities were decreased in liver by 42%, 54% and 44%, respectively. In brain these activities were reduced by 35%, 46% and 65%, respectively. Glutathione reductase remained unchanged. Superoxide production in submitochondrial particles from liver and brain was increased by more than 100% in both tissues. Both reduction of activity of antioxidant enzymes and increased superoxide radical production were prevented by previous injection of 45 mg/kg of nitroarginine, indicating that ammonia induces increased formation of nitric oxide, which in turn reduces the activity of antioxidant enzymes, leading to increased formation of superoxide.

Keywords Hyperammonemia; nitric oxide; nitroarginine; superoxide radical; antioxidant enzymes; ammonia toxicity

INTRODUCTION

Ammonia is a normal product of degradation of proteins and other nitrogenatous compounds. However, at high concentrations, ammonia is neurotoxic, leading to functional disturbances of the central nervous system, which could lead to coma and death. Ureotelic animals detoxicate ammonia by incorporating it into urea, which is eliminated in

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urine. However, when the liver fails, or when blood is shunted past the liver, blood ammonia levels increase and brain function deteriorates: a disorder known as hepatic encephalopathy (e.g. Plum and Hindfelt, 1976; Butterworth, 1992, 1994; Ferenci *et al.*, 1992). The mechanism by which hyperammonemia leads to altered function of the central nervous system remains unclear.

Acute administration of large doses of ammonium salts leads to the rapid death of the animals. Ammonia toxicity was first described more than one century ago in the laboratory of Pavlov (Hahn *et al.*, 1893). However, in spite of much work, the molecular mechanism of acute ammonia toxicity has not been completely clarified. Ammonia intoxication produces marked alterations in brain energy metabolism, including increased lactate, pyruvate and mitochondrial [NAD^+]/[NADH] and decreased cytosolic [NAD^+]/[NADH] (Hindfelt and Siesjö, 1971; Hawkins *et al.*, 1973; Hindfelt *et al.*, 1977; Kosenko *et al.*, 1993) and, at later stages, decreased ATP content (Schenker *et al.*, 1967; Hindfelt *et al.*, 1977; Kosenko *et al.*, 1993, 1994). The changes in energy metabolites could be involved in ammonia-induced coma and death. Ammonia-induced depletion of ATP could be due to increased consumption, decreased synthesis, or both. It has been shown that ammonia-induced depletion of ATP is mediated by activation of the NMDA type of glutamate receptors (Kosenko *et al.*, 1994). Moreover, it has been shown that Na^+/K^+-ATPase activity is increased in brain of rats injected with large doses of ammonia and in cultured neurons treated with glutamate (Kosenko *et al.*, 1994; Marcaida *et al.*, 1996), suggesting increased consumption of ATP. However, ammonia could also decrease ATP content by reducing ATP synthesis in mitochondria. Acute ammonia intoxication impairs mitochondrial function (Kosenko *et al.*, 1997a), which could lead to decreased ATP synthesis and also to increased formation of free radicals. It has been shown that ATP depletion alone is not enough to induce neuronal death (Marcaida *et al.*, 1995). It is also known that free radicals can lead to cell death. We recently found that acute intoxication of rats with 7 mmol/Kg of ammonium acetate, leads to decreased activity of antioxidant enzymes and results in increased formation of superoxide radicals (Kosenko *et al.*, 1997b) in liver and brain. We proposed that these effects of ammonia intoxication would be mediated by increased formation of nitric oxide, which in turn would reduce the activity of antioxidant enzymes and result in increased formation of superoxide radical. To assess this possibility, we have now tested whether previous injection of nitroarginine (an inhibitor of nitric oxide synthase) is able to prevent ammonia-induced alterations. It is shown that nitroarginine prevents completely ammonia-induced reductions of the activities of glutathione peroxidase, catalase and superoxide dismutase (SOD) and increased superoxide formation. It is also shown that the nitric oxide-generating compound sodium nitroprusside added *in vitro* reduces the activity of glutathione peroxidase, catalase and superoxide dismutase, while addition of nitroarginine increases the activity of these enzymes, thus confirming a modulation of these enzymes by nitric oxide.

MATERIALS and METHODS

Animals and experimental conditions

Groups of four-six male Wistar rats weighing 220-250 g were used. One group (Ammonia) was injected i.p. with 7 mmol/kg of ammonium acetate 15 min before killing. Another group (Nitroarginine) was injected with 45 mg/kg of nitro-L-arginine, and was killed 25 min later. A third group (indicated as Nitroarginine + Ammonia) was first injected with nitro-L-arginine, 10 min later with ammonium acetate, and was killed 15 min after ammonia injection. The control group was decapitated 15 min after injection of saline. Immediately after decapitation, brains and livers were removed, and brain and liver mitochondria and submitochondrial particles (SMP) were isolated.

Isolation of liver mitochondria

Livers were quickly removed and perfused with ice-cold 0.9% NaCl to remove blood and homogenized in 9 volumes of homogenization medium (210 mM mannitol with 70 mM sucrose, buffered with 5 mM HEPES, pH 7.4, 1 mM EDTA and 0.5 mg/ml of bovine serum albumin). Mitochondria were isolated from liver homogenate by differential centrifugation. The first centrifugation was performed at 800 x g for 10 min, followed by centrifugation of the resulting supernatant at 9,500 x g for 10 min. The pellet of the second centrifugation was washed twice with the homogenization medium without EDTA and albumin.

Isolation of brain mitochondria

The brain was removed into ice-cold and homogenized as the liver tissue except that homogenization medium was 0.25 M sucrose, 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4. Mitochondria were isolated essentially by the Lai and Clark's method as described by Kosenko *et al.* (1997a). Briefly, the chopped hemispheres in 2.5 ml/g of the isolation medium were homogenized in a Dounce homogenizer. The homogenate was diluted with the isolation medium to a final volume of 5 ml/g tissue and centrifuged twice at 2,000 x g for 3 min and once at 12,000 x g for 8 min to obtain the crude mitochondrial pellet. The pellet was suspended in 1 ml/g tissue of the 3% Ficoll medium (see below), and 3 ml of this suspension was layered onto 12.5 ml of the 6% Ficoll medium and centrifuged at 12,000 x g for 30 min. The loose, fluffy, white top layer of the pellet was removed. The resulting brown pellet was resuspended with 0.5 ml/g of the isolation medium and centrifuged at 12,000 x g for 10 min. The sediment was resuspended to make mitochondrial concentration of about 20 mg of protein per 1 ml. The 6% Ficoll medium contained 6% (w/w) Ficoll, 0.24 M mannitol, 0.06 M sucrose, 0.05 mM K-EDTA, and 10 mM Tris-HCl, pH 7.4. The 3% Ficoll medium was the 6% Ficoll medium diluted with glass-distilled water. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of submitochondrial particles (SMP) from rat liver and brain

Mitochondrial pellets were washed two times and suspended in 10 mM Tris-HCl, pH 7.4, at 10 mg protein/ml and allowed to stay at 0°C for 15 min with stirring. The suspension was centrifuged at 20,000 x g and 4°C for 20 min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, at 10 mg protein/ml and frozen/thawed three times at liquid

nitrogen/room temperatures. The suspension was centrifuged at 2,000 x g for 10 min. The supernatant was centrifuged at 144,000 x g and 4°C for 30 min, and the firmly packed pellet was washed three times with 10 mM Tris-HCl, pH 7.4 and stored in the same medium at 20°C.

Determination of enzyme activities

Mitochondria were disrupted by osmotic shock in 10 mM phosphate buffer (pH 7.4, 10 min at 4°C) and three freezing-thawing cycles. The suspension obtained was centrifuged for 20 min at 140,000 x g, and the supernatant was used as the source of enzymes. Catalase activity was determined without disruption of mitochondria in the presence of 1% Triton X-100.

Activity of glutathione reductase was measured by following the decrease in absorbance due to the oxidation of NADPH by oxidized glutathione (Goldberg and Spooner, 1984). Glutathione peroxidase activity was measured in coupled reaction as described by Lawrence and Burk (1976). We used 5 mM GSH instead of 1 mM concentration because the Km values for GSH were found to be approximately of 2.2 mM for the liver enzyme and 2.5 mM for the brain enzyme (unpublished data).

Catalase was assayed with hydrogen peroxyde by measuring the decrease in absorption at 240 nm as described by Aebi (1984). The enzyme activity was expressed in terms of the first-order reaction rate constant (Cohen *et al.*, 1970), i. e. sec^{-1} per mg of protein. SOD activity was determined by the Beauchamp and Fridovich's method (1971) by the inhibition of the reduction of nitrotetrazolium blue (NTB) in the presence of xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the rate of NTB reduction.

Measurement of superoxide radical production in SMP

Adrenochrome method. The superoxide-dependent oxidation of epinephrine to adrenochrome was measured by the Boveris' procedure (1984).

Dichlorophenolindophenol method. The superoxide-dependent reduction of dichlorophenolindophenol (DCIP) was measured by the method of Forman and Kennedy (1976).

RESULTS

Injection of 7 mmol/kg of ammonium acetate to rats produced a significant decrease in the activity of superoxide dismutase in mitochondria from liver (by 54%, Table 1) and brain (by 46%, 2).

Injection of nitroarginine alone to rats did not affect significantly the activity of superoxide dismutase in liver mitochondria (Table 1); however, nitroarginine injection increased strongly (more than 4-fold) the activity of superoxide dismutase in brain mitochondria (Table 2).

When rats were injected with nitroarginine and ammonia, the activity of superoxide dismutase in liver mitochondria was simliar to that of control rats and of rats injected only with nitroarginine (Table 1). This indicates that inhibition of nitric oxide synthase by nitroarginine prevents completely the reduction in the activity of superoxide dismutase induced in liver by ammonia intoxication.

Table 1. Injection of ammonia to rats reduces the activity of antioxidant enzymes in liver mitochondria. Prevention of the effect by nitroarginine.

Enzyme	Control	Ammonia	Nitroarginine	Nitroarginine + Ammonia
Superoxide dismutase (U/min x mg protein)	7.3 ± 0.5	3.3 ± 0.5*	8.5 ± 0.6	8.0 ± 0.5
Catalase (sec^{-1}/mg protein)	0.70 ± 0.01	0.39 ± 0.05*	0.75 ± 0.02	0.71 ± 0.01
Glutathione peroxidase (nmol/min x mg protein)	303 ± 15	174 ± 8*	548 ± 30*	494 ± 25*
Glutathione reductase (nmol/min x mg protein)	55 ± 7	50 ± 5	52 ± 5	67 ± 7

Groups of four-six rats were used. One group (Ammonia) was injected i.p. with 7 mmol/kg of ammonium acetate 15 min before killing. Another group (Nitroarginine) was injected with 45 mg/kg of nitro-L-arginine, and was killed 25 min later. The group indicated as (Nitroarginine + Ammonia) was first injected with nitro-L-arginine, 10 min later with ammonium acetate, and was killed 15 min after ammonia injection: The control group was decapitated 15 min after injection of saline. Immediately after decapitation, livers were removed, and liver mitochondria were isolated and assayed for enzyme activities as indicated in Methods. Values are the mean ± standard deviations of triplicate measurements from 4-6 rats per group. * Significantly different from controls.

The activity of superoxide dismutase in brain mitochondria from rats injected with nitroarginine and ammonia was 6-fold higher than in rats injected only with ammonia and 3-fold higher than in control rats (Table 2). However, the activity was slightly less (72%) than in rats injected only with nitroarginine.

Acute ammonia intoxication reduced the activity of catalase by 44% in liver mitochondria (Table 1) and by 65% in brain mitochondria (Table 2). As is the case for superoxide dismutase, injection of nitroarginine alone did not affect catalase activity in liver (Table 1), but increased it by 76% in brain (Table 2).

Injecting nitroarginine before ammonia prevented completely the reduction of catalase activity induced by ammonia in liver (Table 1) and brain (Table 2). In both tissues, the activity in rats injected with nitroarginine and ammonia was similar to that of rats injected only with nitroarginine.

Table 2. Injection of ammonia to rats reduces the activity of antioxidant enzymes in brain mitochondria. Prevention of the effect by nitroarginine.

Enzyme	Control	Ammonia	Nitroarginine	Nitroarginine + Ammonia
Superoxide dismutase (U/min x mg protein)	3.6 ± 0.1	1.9 ± 0.1*	16.0 ± 0.4*	11.5 ± 0.9*
Catalase (sec^{-1}/mg protein x 104)	3.4 ± 0.2	1.2 ± 0.2*	6.0 ± 0.7*	5.1 ± 0.9
Glutathione peroxidase (nmol/min x mg protein)	54 ± 9	35 ± 8*	115 ± 12*	83 ± 5*
Glutathione reductase (nmol/min x mg protein)	36 ± 6	33 ± 6	54 ± 5	40 ± 5

Groups of four-six rats were used. One group (Ammonia) was injected i.p. with 7 mmol/kg of ammonium acetate 15 min before killing. Another group (Nitroarginine) was injected with 45 mg/kg of nitro-L-arginine, and was killed 25 min later. The group indicated as (Nitroarginine + Ammonia) was first injected with nitro-L-arginine, 10 min later with ammonium acetate, and was killed 15 min after ammonia injection: The control group was decapitated 15 min after injection of saline. Immediately after decapitation, brains were removed, and mitochondria were isolated and assayed for enzyme activities as indicated in Methods. Values are the mean ± standard deviations of triplicate measurements from 4-6 rats per group. * Significantly different from controls.

Injection of ammonia reduced the activity of glutathione peroxidase in mitochondria from liver (by 42%, Table 1) and brain (by 35%, Table 2). Injection of nitroarginine alone increased glutathione peroxidase activity both in liver (by 82%, Table 1) and brain (by 113%, Table 2).

When rats were injected with nitroarginine and ammonia, the activity of glutathione peroxidase in liver mitochondria was higher than in rats injected with ammonia (284%) and than in control rats (163%), and similar to that of rats injected only with nitroarginine (Table 1). In mitochondria from brains of these rats, the activity of glutathione peroxidase was higher than in rats injected with ammonia (237%) and than in control rats (154%), but less (72%) than in rats injected only with nitroarginine.

Glutathione reductase activity remained unaltered following ammonia and/or nitroarginine injection (Tables 1 and 2).

As shown in Table 3, superoxide radical production was increased in liver and brain submitochondrial particles of rats injected with ammonium acetate. When the adenochrome formation method was used for measurement of superoxide radical formation, there was a 95% increase when measured in liver SMP from rats treated with ammonia, while it remained undetectable in brain SMP. Using the reduction of DCIP method, there was an 85% increase of superoxide radical production in liver SMP and of 3-times in brain SMP from rats treated with ammonia (Table 3).

Table 3. Injection of ammonia to rats increases the formation of superoxide radical in liver and brain submitochondrial particles. Prevention of the effect by nitroarginine.

Superoxide formation	Control	Ammonia	Nitroarginine	Nitroarginine +Ammonia
Adrenochrome formation: (nmol/min.mg protein)				
Liver SMP	3.2 ± 0.3	6.2 ± 0.5 *	3.4 ± 0.2	3.3 ± 0.3
(% inhibition by SOD)	(90)	(90)	(87)	(90)
2,6-dichlorophenolindophenol (nmol/min.mg protein)				
Liver SMP	2.2 ± 0.1	4.0 ± 0.2 *	2.0 ± 0.3	2.4 ± 0.3
(% inhibition by SOD)	(43)	(41)	(40)	(40)
Brain SMP	1.4 ± 0.2	4.2 ± 0.3 *	1.2 ± 0.3	1.3 ± 0.4
(% inhibition by SOD)	(82)	(79)	(80)	(80)

Groups of four-six rats were used. One group (Ammonia) was injected i.p. with 7 mmol/kg of ammonium acetate 15 min before killing. Another group (Nitroarginine) was injected with 45 mg/kg of nitro-L-arginine, and was killed 25 min later. The group indicated as (Nitroarginine + Ammonia) was first injected with nitro-L-arginine, 10 min later with ammonium acetate, and was killed 15 min after ammonia injection: The control group was decapitated 15 min after injection of saline. Immediately after decapitation, livers and brains were removed, and mitochondria and submitochondrial particles (SMP) were isolated and assayed for generation of superoxide radical as indicated in Methods. Values are the mean ± standard deviations of triplicate measurements from 4-6 rats per group. * Significantly different from controls.

Loschen *et al.* (1974) reported that liver succinate dehydrogenase produces superoxide while brain succinate dehydrogenase does not (Sorgato *et al.*, 1974). In our experiments, although rat brain SMP were deprived of endogenous SOD activity, no oxidation of adrenaline was detected in rat brain SMP supplemented with succinate and antimycin A while in liver SMP about 3.2 nmoles of adrenochrome/min/mg protein were formed (Table 3). Addition of SOD (3 mg/ml) produced an almost complete inhibition of adrenochrome formation by liver SMP indicating that the oxidation of adrenaline was caused by superoxide radicals (McCord and Fridovich, 1969).

Using the method of Forman and Kennedy (1976) based on the ability of dihydroorotate to reduce dichlorophenolindophenol (DCIP) in the presence of cyanide, we found that the rate of superoxide radical formation in rat brain SMP under above conditions was 1.4 nmol/min/mg protein (Table 3) and that the addition of cyanyde-insensitive Mn^{2+}-SOD from E. coli inhibited DCIP reduction by 80%. Higher levels of SOD did not induce further inhibition. Injection of ammonia produced a two-fold increase in superoxide formation in brain.

Injection of nitroarginine alone did not affect the formation of superoxide in liver or brain (Table 3).

Injection of nitroarginine before intoxication with ammonia prevents completely the increase in superoxide formation induced by ammonia (Table 3).

The above results suggest that the activity of SOD, catalase and glutathione peroxidase in brain mitochondria are modulated by nitric oxide. To assess this possibility we tested whether addition of sodium nitroprusside (SNP), a nitric oxide-generating agent, to brain mitochondria reduces the activity of these enzymes. As shown in Figure 1, SOD is very sensitive to SNP. Addition of 2.5 and 5 mM SNP reduced immediately the activity of SOD by 20% and 59%, respectively. Catalase and glutathione peroxidase were less sensitive to SNP. Both enzymes were inhibited (by 34% and 30%, respectively) after a short preincubation with 200 mM SNP.

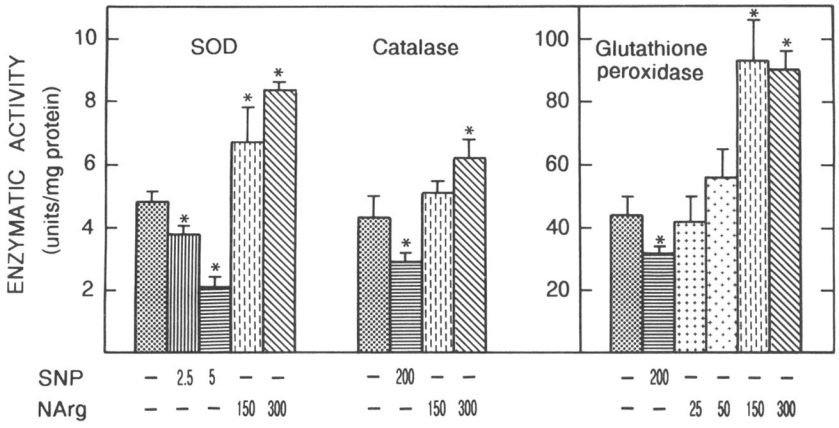

Figure 1. Sodium nitroprusside decreases and nitroarginine increases the activities of SOD, catalase and glutathione peroxidase in brain mitochondria. Brain mitochondria were isolated and enzymatic activities were assayed as indicated in the Methods section. For assessing the effects of the nitric oxide generating compound sodium nitroprusside (SNP), it was added to the assay mixture at the concentrations indicated (in mM) in the Figure. For the assay of SOD, SNP (2.5 or 5 mM) was added at the beginning of the assay. For the assays of catalase and of glutathione peroxidase, SNP (200 mM) was added 10 or 30 min, respectively, before starting the assay. Nitroarginine at the indicated concentrations (in mM) was added 5 min before starting the assay. Activities are expressed in the same units indicated in Methods and in Table 2. Values are the mean ± standard deviations of triplicate samples from two different experiments. Values which are significantly different from controls ($p<0.01$) are indicated by asterisks.

To assess whether there is a tonic inhibition of the enzymes by endogenous nitric oxide, we tested if addition of nitroarginine *in vitro* to brain mitochondria increases the activity of SOD, catalase and glutathione peroxidase. As shown in Figure 1, addition of nitroarginine increased significantly the activity of all these enzymes.

SOD activity was increased by 37% and 71% when mitochondria were incubated with 150 and 300 mM nitroarginine, respectively. Catalase activity was increased by 16% and 41% by 150 and 300 mM nitroarginine, respectively. Glutathione peroxidase activity was increased by 111% and 105% by 150 and 300 mM nitroarginine, respectively.

The decreased activity of the antioxidant enzymes in the presence of the nitric oxide-generating agent could be due to a direct effect of nitric oxide on the enzymes or to an indirect effect mediated by other components in the mitochondria. To assess whether nitric oxide is able to inhibit directly the activity of catalase, we tested the effect of SNP added in vitro to a commercial preparation of purified catalase. SNP inhibited in a dose-dependent manner the activity of purified catalase (not shown), thus indicating that catalase is inhibited directly by nitric oxide.

DISCUSSION

The results reported confirm that acute intoxication with large doses of ammonia reduces the activity of antioxidant enzymes in liver and brain mitochondria and results in increased formation of superoxide radical. This is in agreement with a recent report (Kosenko *et al.*, 1997b) using the same regime of ammonia administration and indicate that acute ammonia intoxication results in oxidative stress.

Acute and chronic hyperammonemia in mice lead to increased lipid peroxidation in liver and brain, reflecting an oxidative stress situation (O'Connor and Costell, 1990). Some effects of milder hyperammonemia in experimental animals or in human patients with liver failure on antioxidant enzymes have been reported. A decrease of glutathione-related enzymes and catalase activities has been shown in liver of rats treated with thioacetamide, an experimental model of hepatic failure and hyperammonemia. SOD increased progressively in thioacetamide-treated rats (Sanz *et al.*, 1995). SOD and catalase activities were decreased in liver of rats teated with carbon tetrachloride, another animal model producing moderate hyperammonemia (Nadkarni and D'Souza, 1988). It has not been clarified whether the alterations in antioxidant enzymes in thioacetamide- or carbon tetrachloride-treated rats are a direct effect of ammonia or are due to liver cirrhosis or other alterations induced by the hepatotoxins.

The antioxidant enzymes have been also measured in human patients with different liver diseases. Both glutathione peroxidase and glutathione reductase remain unaltered in liver of patients with different liver diseases (Corrocher *et al.*, 1980). SOD has been reported to be decreased in erythrocytes of patients with liver cirrhosis (Ozenirler *et al.*, 1994).

Acute intoxication with large doses of ammonia as used in the present work leads to higher ammonia levels in blood and to the rapid (half-life ca. 30 min) death of the animals. So that it should be expected that such acute intoxication would lead to more drastic effects than the milder hyperammonemia present in the above reports.

The results reported show that ammonia intoxication reduces the activity of antioxidant enzymes. A diminished SOD activity would lead to decreased superoxide elimination. Impaired activity of glutathione peroxidase and catalase would lead to decreased elimination of peroxide. The decrease in these enzyme activities revealed a diminished protection of liver and brain against free radical damage following acute ammonia intoxication. Ammonia also increases the production of superoxide radicals (Table 3). Both effects would

lead to oxidative stress, leading to hepatocellular and neuronal damage, which could be involved in the mechanism by which ammonia intoxication leads to the death of the animal.

The above results show that acute ammonia intoxication causes a rapid alteration in the activities of glutathione peroxidase, superoxide dismutase and catalase, but not of glutathione reductase. The activities are reduced 15 min after injection of ammonia.

We propose, on the bases of the previous studies discussed below, that the ammonia-induced inhibition of antioxidant enzymes in brain is mediated by activation of NMDA receptors, of nitric oxide synthase and formation of nitric oxide, which inhibits the enzymes.

Acute ammonia intoxication leads to a rapid activation of NMDA receptors, as indicated by the following findings:
Firstly, acute ammonia intoxication, produced exactly as in the present study, leads to rapid mortality. Ammonia-induced death of animals is prevented by ten different antagonists of NMDA receptors (Hermenegildo *et al.*, 1996), indicating that activation of these receptors mediates the toxic effects of ammonia. This activation of NMDA receptors occurs very rapidly; since ammonia-induced death of animals, which occurs after activation of NMDA receptors, takes place in 20-30 min (Miñana *et al.*, 1988).

Secondly, acute intoxication with ammonia, using conditions identical to those used in the present study, also produces, rapidly, the following effects: depletion of brain ATP (Kosenko *et al.*, 1993), which occurs in less than 10 min; proteolysis of MAP-2 in brain (Felipo *et al.*, 1993), and activation of Na^+/K^+-ATPase (Kosenko *et al.*, 1994). All these effects are completely prevented by MK-801, a selective antagonist of NMDA receptors (Kosenko *et al.*, 1994; Felipo *et al.*, 1993).

These observations clearly indicate that acute ammonia intoxication leads to excessive activation of NMDA receptors, which occur in less than 10 min after injection of ammonia.

It is well known that activation of NMDA receptors leads to the opening of the ion channel, leading to increased concentration of intracellular free calcium in the post-synaptic neuron with concomittant activation of nitric oxide synthase and increased formation of nitric oxide.

An increase in nitric oxide formation following ammonia intoxication is supported by the fact that ammonia-induced death of animals is markedly attenuated by nitroarginine, an inhibitor of nitric oxide synthase (Kosenko *et al.*, 1995). This indicates that acute ammonia intoxication leads to excessive activation of NMDA receptors, leading to activation of neuronal nitric oxide synthase, increasing the formation of nitric oxide, which contributes to the toxic effects of ammonia.

Nitric oxide can reduce the activity of antioxidant enzymes as supported by the following findings: It has been shown that nitric oxide inhibits the activity of catalase (Brown, 1995; Mohazzab *et al.*, 1996; Farias-Eisner, 1996) and glutathione peroxidase (Asahi *et al.*, 1995). Nitric oxide inhibits the mitochondrial respiratory chain in astrocytes, contributing to the formation of free radicals (Bolaños *et al.*, 1994).

The results reported here clearly support the notion that the activity of these enzymes is modulated by nitric oxide both *in vivo* and *in vitro*. As shown in Tables 1 and 2, injection of nitroarginine alone, to inhibit nitric oxide synthase, results in a strong increase in the activities of superoxide dismutase (more than 4-fold) and of catalase (by 76%) in brain and of glutathione peroxidase both in liver (by 81%) and brain (by 113%). This indicates that nitric oxide could exert a tonic inhibition of these enzymes *in vivo*, which would be removed when nitric oxide synthase is inhibited by nitroarginine. Moreover, the results shown in Figure 1 clearly demonstrate that the nitric oxide-generating agent SNP inhibits strongly SOD and less potently catalase and glutathione peroxidase. Also, nitroarginine added to brain mitochondria increases the activity of SOD, catalase and glutathione peroxidase, suggesting a tonic inhibition of these enzymes by nitric oxide.

The results reported in Tables 1-3 also indicate that the reduction in the activities of superoxide dismutase, catalase and glutathione peroxidase and the subsequent increase in superoxide, induced by acute ammonia intoxication in rats, is due to the activation of nitric oxide synthase. This is supported by the observation that injection of nitroarginine prevents completely the reduction of the enzyme activities and the increase in the formation of superoxide induced by ammonia.

The NMDA receptor-mediated formation of nitric oxide would explain the effects of ammonia in brain, but not in tissues lacking these receptors such as liver. We have shown that ammonia may lead to increased nitric oxide formation by an additional mechanism, independent of activation of NMDA receptors (Kosenko *et al.*, 1995). In fact, it has been reported that ammonia may generate nitric oxide in aorta and liver of the rat (Thomas and Ramwell, 1988; Wettstein *et al.*, 1994). This additional mechanism of ammonia-induced formation of nitric oxide would be responsible for inhibition of antioxidant enzymes in liver and other tissues. This is supported by the fact that nitroarginine also prevents ammonia-induced changes in antioxidant enzymes and superoxide formation in liver. It is also possible that this NMDA receptor-independent mechanism of induction of nitric oxide formation by ammonia could be responsible for the inhibition of antioxidant enzymes in brain.

Further studies to clarify the effects of nitric oxide-induced alterations in these enzymes would contribute to understanding the mechanisms of ammonia toxicity and of glutamate neurotoxicity.

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