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Superoxide Production and Antioxidant Enzymes in Ammonia Intoxication in Rats

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Injection of large doses of ammonium salts lead to the rapid death of animals. However, the molecular mechanisms involved in ammonia toxicity remain to be clarified. We have tested the effect of injecting 7 mmol/kg of ammonium acetate on the production of superoxide and on the activities of some antioxidant enzymes in rat liver, brain, erythrocytes and plasma. Glutathione peroxidase, superoxide dismutase and catalase activities were decreased in liver and brain (both in cytosolic and mitochondrial fractions) and also in blood red cells, while glutathione reductase activity remained unchanged. Superoxide production in submitochondrial particles from liver and brain was increased by more than 100% in both tissues. Both diminished activity of antioxidant enzymes and increased superoxide radical production could lead to oxidative stress and cell damage, which could be involved in the mechanism of acute ammonia toxicity.

Keywords: Hyperammonemia, superoxide radical, antioxidants enzymes, brain, ammonia toxicity, free radicals

INTRODUCTION

Ammonia is present in living organisms as a product of degradation of proteins and other compounds. At high levels, ammonia is neurotoxic, leading to functional disturbances of the central nervous system, which can lead to coma and death. When liver fails, or when blood is shunted past the liver, blood ammonia levels increase and brain function deteriorates, a disorder known as hepatic encephalopathy.[1] The mechanism by which liver failure or hyperammonemia per se lead to disturbances in brain function remains unclear.

Acute administration of large doses of ammonium salts leads to rapid death of the animals. The molecular mechanism of acute ammonia toxicity has not been eludicated in spite of much work since the first report on ammonia toxicity more than one century ago.[2] Ammonia intoxication leads to astrocyte and brain swelling. Astrocyte swelling activates signal transduction processes and alters cellular metabolism.[3] Disturbance of cell volume homeostasis in the brain has been

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suggested to be an early pathogenetic event in ammonia toxicity and hepatic encephalopathy.[4] Acute ammonia intoxication produces marked alterations in brain energy metabolism, including increased lactate, pyruvate and mitochondrial [NAD^+]/[NADH] and decreased cytosolic [NAD^+]/[NADH][5–8] and, in a later step, decreased ATP content.[7–9] It is considered that the changes in energy metabolites would be involved in the origin of ammonia-induced coma and death. Ammonia-induced depletion of ATP could be due to increased consumption, decreased synthesis, or both. Na^+/K^+-ATPase activity is increased in brain of rats injected with large doses of ammonia,[9] suggesting increased consumption of ATP. However, ammonia could also decrease ATP content by reducing ATP synthesis in mitochondria. We have recently shown that acute ammonia intoxication impairs mitochondrial function.[10] This could lead to decreased ATP synthesis and also to increased formation of free radicals. It has been shown that ATP depletion alone is not sufficient to induce neuronal death.[11] It is also known that free radicals can lead to cell death. We therefore considered of interest to test whether acute administration to rats of a dose of ammonia (7 mmol/kg) which interferes with mitochondrial function leads to increased formation of superoxide radicals and/or to decreased activity of antioxidants enzymes. We have measured the effects of ammonia intoxication on the activities of glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase (SOD) in liver and brain (both cytosolic and mitochondrial fractions) and in erythrocytes and plasma. Superoxide formation by liver and brain submitochondrial particles (SMP) from control and ammonia-intoxicated rats was also measured.

MATERIALS AND METHODS

Animals and Experimental Conditions

Male Wistar rats weighing 220–250 g were used. For the ammonia group, rats were injected intraperitoneally with 7 mmol/kg of ammonium acetate. Control rats received an injection of saline. The animals were killed by cervical dislocation 15 min after injection.

Isolation of Mitochondria and Cytosol from Liver and Brain

Livers were homogenized in 9 volumes of 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). Mitochondrial and cytosolic fractions were isolated by differential centrifugation. The brain was 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. Mitochondrial and cytosolic fractions were isolated essentially by the Lai and Clark's method as described by Kosenko *et al.*.[10] Protein was determined by the method of Lowry *et al.*[12] Submitochondrial particles (SMP) from liver and brain were prepared essentially as described by Senior.[13]

Preparation of Erythrocytes

Rat blood (0.25 ml) was mixed with 0.25 ml heparin (10 units per ml of blood) and centrifuged at 1,000 × g for 15 min. Red sediment was washed in 0.9% NaCl twice under the same conditions.

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 was centrifuged 20 min at 140,000 × 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. Erythrocytes were lysed by incubating at 4°C in 12 mM triethanolamine buffer (pH 7.5) with 0.02% saponin. 15 min later, the incubation mixture was centrifuged at 1,000 × g for 15 min and the insoluble material was discarded.

3-D-hydroxybutyrate dehydrogenase and succinate dehydrogenase activities in mitochondrial preparations were determined as described earlier.[14] Activity of glutathione reductase was measured as in.[15] Glutathione peroxidase activity was measured as by Lawrence and Burk.[16] We used 5 mM GSH instead of 1 mM concentration because the K_m 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 as described by Aebi.[17] The enzyme activity was expressed in terms of the first-order reaction rate constant,[18] i.e. sec^{-1} (per mg of protein or 1 ml of cells or plasma). SOD activity was determined by Beauchamp and Fridovich's method.[19] 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 Rat Liver SMP

Adrenochrome method The superoxide-dependent oxidation of epinephrine to adrenochrome was measured by Boveris' procedure.[20]

Dichlorophenolindophenol method The superoxide-dependent reduction of dichlorophenolindophenol (DCIP) was measured by the method of Forman and Kennedy.[21]

RESULTS AND DISCUSSION

Ammonium acetate injection produced a significant decrease of glutathione peroxidase activity, both in rat liver (24% decrease in cytosol and 35% in the mitochondrial fraction) and brain (38% decrease in cytosol and 47% in mitochondrial fraction) and also in red blood cells (25% decrease). Glutathione reductase activity remained unaltered following ammonia injection (Table I). Superoxide dismutase and catalase activities were also decreased by ammonium injection. SOD activity decreased in liver (32% in cytosol and 41% in mitochondrial fraction), brain (40% in cytosol and 37% in mitochondrial fraction) and red blood cells (69% decrease). Catalase activity was also decreased in liver (42% in cytosol and 27% in mitochondrial fraction), brain (52% in cytosol and 58% in mitochondrial fraction) and red blood cells (29% decrease) but was increased in plasma (88% increase), as shown in Table II.

TABLE I Activities of glutathione peroxidase and glutathione reductase in cytosolic fractions of liver and brain, liver and brain mitochondria, and in erythrocytes and plasma from control and ammonium injected rats

Preparation	Control	Ammonium acetate
Glutathione peroxidase		
Liver cytosol (nmol/min × mg protein)	1370 ± 160	1040 ± 100*
Brain cytosol (nmol/min × mg protein)	130 ± 30	81 ± 9*
Liver mitochondria (nmol/min × mg protein)	328 ± 22	212 ± 32*
Brain mitochondria (nmol/min × mg protein)	30 ± 4	16 ± 0.8*
Erythrocytes (μmol/min × ml of cells)	125 ± 23	94 ± 23*
Plasma (nmol/min × ml)	5330 ± 200	5690 ± 600
Glutathione reductase		
Liver cytosol (nmol/min × mg protein)	109 ± 10	103 ± 6
Brain cytosol (nmol/min × mg protein)	45 ± 7	41 ± 5
Liver mitochondria (nmol/min × mg protein)	51 ± 7	51 ± 11
Brain mitochondria (nmol/min × mg protein)	19 ± 1	14 ± 2
Erythrocytes (μmol/min × ml of cells)	67 ± 12	66 ± 16
Plasma (nmol/min × ml)	38 ± 5	38 ± 4

Results are mean ± SE for 7–16 measurements on preparations from six rats. *$P < 0.05$ as compared with controls.

TABLE II Activities of superoxide dismutase and catalase in cytosolic fractions of liver and brain, liver and brain mitochondria, and in erythrocytes and plasma from control and ammonium injected rats

Preparation	Control	Ammonium acetate
Superoxide dismutase		
Liver cytosol (U/min × mg protein)	53 ± 4.6	36 ± 3*
Brain cytosol (U/min × mg protein)	10 ± 0.9	6 ± 0.9*
Liver mitochondria (U/min × mg protein)	8 ± 0.9	4.7 ± 0.8*
Brain mitochondria (U/min × mg protein)	4.3 ± 0.0	2.7 ± 0.1
Erythrocytes (U/min × ml of cells)	932 ± 62	286 ± 90*
Plasma (U/min × ml)	1.5 ± 0.1	1.6 ± 0.1
Catalase		
Liver cytosol (sec^{-1}/mg protein)	0.31 ± 0.03	0.18 ± 0.016*
Brain cytosol (sec^{-1}/mg protein × 10^4)	11.4 ± 1.8	5.5 ± 0.42*
Liver mitochondria (sec^{-1}/mg protein)	1.1 ± 0.08	0.80 ± 0.09*
Brain mitochondria (sec^{-1}/mg protein × 10^4)	3.7 ± 1.8	1.55 ± 0.15*
Erythrocytes (sec^{-1}) ml of cells)	24 ± 2.2	17 ± 1.7*
Plasma (sec^{-1}/ml × 10^5)	1.6 ± 0.1	3.0 ± 0.8*

Results are mean ± SE for 7–16 measurements on preparations from six rats except of n = 3 for brain mitochondria. *P. < 0.05 as compared with controls.

As far as we know, there are no data in the literature about the effects of acute ammonia intoxication on the activity of these enzymes. Acute and sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress situation.[22] Some effects of milder hyperammonemia in experimental animals or in human patients with liver failure on the above 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.[23] SOD and catalase activities were decreased in liver of rats teated with carbon tetrachloride, another animal model producing moderate hyperammonemia.[24] These 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.[25] SOD has been reported to be decreased in erythrocytes of patients with liver cirrhosis.[26]

It should be noted that acute intoxication with large doses of ammonia as used in the present work leads to higher ammonia levels in blood and to the rapid death of the animals. Following injection of ammonia, the rats die at 28 ± 9 min.

As shown in Table III, superoxide radical production was increased in liver and brain submitochondrial particles of rats injected with ammonium acetate. When the adrenochrome formation method was used for measurement of superoxide radical formation, there was an increase of 2.6-times when measured in liver SMP from rats treated with ammonia, while remained undetectable in brain SMP. Using the reduction of DCIP method, there was an increase of superoxide radical production of 1.8-times in liver SMP and 2.0-times in brain SMP from rats treated with ammonia (Table III).

Loschen *et al.*[27] reported that, while superoxide is produced by liver succinate dehydrogenase, it was not formed by succinate dehydrogenase in rat brain.[28] 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

TABLE III Adrenochrome and DCPIP reduction by liver and brain SMP from normal and ammonium injected rats and its inhibition by added SOD

Preparation	Superoxide formation nmol of product/min.mg protein		Inhibition by SOD, %	
	Control	Hyperammonemia	Control	Hyperammonemia
		Adrenochrome formation*		
Liver SMP	2.5 ± 0.1	6.6 ± 0.6	90	88
Brain SMP		Undetectable		
		Reduction of 2,6-dichlorophenol indophenol (DCPIP)		
Liver SMP	3.0 ± 0.1	5.4 ± 0.2	35	40
Brain SMP	1.9 ± 0.1	3.9 ± 0.2	78	81

Experimental conditions are described in the Methods Section. Inhibition of the adrenochrome formation and DCIP reduction was measured in the presence of 3 μg/ml of cyanide-insensitive Mn^{2+}-SOD from Escherichia coli (Forman and Kennedy, 1974); two-fold higher levels of SOD did not increase the inhibitory effect. Means ± S.D. for 4 separate experiments are given, with 3–5 measurements per each experiment.

antimycin A while in liver SMP about 2.5 nmoles of adrenochrome/min/mg protein were formed (Table III). Addition of SOD (3 mg/ml) produced an almost complete suppression of adrenochrome formation by liver SMP indicating that the oxidation of adrenaline was caused by superoxide radicals.[29]

Using the method of Forman and Kennedy[21] 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 the above conditions was 1.9 nmol/min/mg protein (Table III) and that the addition of cyanide-insensitive Mn^{2+} -SOD from E. coli inhibited DCIP reduction by 70%. Higher levels of SOD did not induce further inhibition. Injection of ammonia produced a two-fold increase in superoxide formation in brain.

The results reported show that ammonia intoxication diminishes 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 hydroperoxides. The decrease in these enzyme activities revealed a diminished protection of liver, brain and red blood cells against free radical damage following acute ammonia intoxication. Ammonia also increases the production of superoxide radicals. 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 animal death.

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 lowered 15 min after injection of ammonia and the greatest inhibition is found in the brain.

We propose that ammonia-induced inhibition of antioxidant enzymes is mediated by activation of NMDA receptors, of nitric oxide synthase and formation of nitric oxide, which inhibits the enzymes.

The following facts support that acute ammonia intoxication leads to a rapid activation of NMDA receptors:

1) Injection of lethal doses of ammonia leads to the death of mice and rats in less than 30 min.[30] Ammonia-induced death of animals is prevented by antagonists of NMDA receptors.[31]
2) Injection of 7 mmol/kg of ammonium acetate to rats, as in the preset work, leads to depletion

of brain ATP in less than 10 min.[8] Ammonia-induced depletion of ATP is prevented by blocking the NMDA receptor with MK-801.[9]

3) Injection of ammonia to rats as in the present work induces a proteolisis of MAP-2 in brain, which is prevented by MK-801.[32]
4) Acute ammonia intoxication leads to a rapid activation of brain Na^+/K^+-ATPase, which is prevented by MK-801.[9]

All these results indicate that acute ammonia intoxication, as that used in the present work, leads to excessive activation of NMDA receptors in brain in less than 10 min. This activation of NMDA receptors leads to increased intracellular Ca^{2+} which in turn activates neuronal nitric oxide synthase, increasing the formation of nitric oxide, which contributes to the toxic effects of ammonia. This is supported by the fact that nitroarginine, an inhibitor of nitric oxide synthase, also prevents ammonia-induced death of animals and depletion of ATP.[33] This indicates that ammonia intoxication increases brain nitric oxide, which in turn plays a role in the mediation of ammonia-induced depletion of ATP and death of animals. As ATP depletion occurs in less than 10 min after ammonia injection, increased formation of nitric oxide should also occur in less than 10 min.

This increase in nitric oxide would be responsible for the rapid lowering of antioxidant enzyme activities. Nitric oxide can decrease these activities both directly and/or indirectly. It has been shown that nitric oxide inhibits directly the activity of catalase[34–36] and glutathione peroxidase.[37] Moreover, nitric oxide inhibits the mitochondrial respiratory chain in astrocytes, contributing to the formation of free radicals.[38] Also, increased nitric oxide results in the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) and to mixed glutathione disulfides (GSSR), resulting in depletion of GSH and increased free radicals.[39] Nitric oxide is also able to react with superoxide to form peroxynitrite, a potent damaging oxidant; nitric oxide captures superoxide three times faster than SOD-1, then interfering with its elimination and leading to formation of peroxynitrite.[40]

Ammonia-induced activation of NMDA receptors under the conditions used in the present work also induces the activation of calpain, a Ca^{2+}-dependent protease, resulting in the rapid proteolysis of the microtubule-associated protein MAP-2 in rat brain.[32] It has been proposed that activation of calpain would also induce the proteolysis of xanthine dehydrogenase, leading to activation of xanthine oxidase and to increased formation of free radicals.[41]

We therefore propose that the ammonia-induced inhibition of antioxidant enzymes is mediated by activation of NMDA receptors, of nitric oxide synthase and formation of nitric oxide, which inhibits the enzymes. Nitric oxide would also contribute to increased oxidative damage by depleting GSH and by forming peroxynitrite. Moreover, ammonia-induced activation of NMDA receptors leads to activation of calpain, which would activate xanthine oxidase, thus contributing to increased oxidative damage.

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.[33] In fact, it has been reported that ammonia may generate nitric oxide in aorta and liver of the rat.[42–43] This additional mechanism of ammonia-induced formation of nitric oxide would be responsible for inhibition of antioxidant enzymes in liver and other tissues.

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References

[1] Plum, F. and Hindfelt, B. (1976). The neurological complications of liver disease. In *Metabolic and Deficiency Diseases of the Nervous System, Part 1, 27* (eds. P. J. Vinken, G. W. Bruyn and H. L. Klawans), Elsevier Publ. Co., New York, pp. 349–377.

[2] Hahn, M., Massen, O., Nencki, M. and Pavlov, I. (1893). Die Eck'sche Fistel zwischen der unteren Hohlvene und der Pfortader und ihre Folgen für den Organismus. *Archiv für Experimentelle Pathologie und Pharmakologie*, **32**, 161–210.

[3] Häussinger, D., Warskulat, V. and Schliess, F. (1997). Osmosignalling and osmolytes in liver and astrocytes. In *Advances in Cirrhosis, Hyperammonemia and Hepatic Encephalopathy* (eds. V. Felipo and S. Grisolía) Adv. Exp. Med. Biol. **420**, pp. 195–217.

[4] Häussinger, D., Laubenberger, J., Dahl, S. v, Ernst, T., Bayer, S., Langer, M., Gerok, W. and Henning, J. (1994). Proton magnetic resonance spectroscopy studies on human brain myoinositol in hypo-osmolarity and hepatic encephalopathy. *Gastroenterology*, **107**, 1475–1480.

[5] Hindfelt, B. and Siesjö, B. K. (1971). Cerebral effects of acute ammonia intoxication. *Scandinavian Journal of Clinical & Laboratory Investigation*, **28**, 365–374.

[6] Hawkins, R. A., Miller, A. L., Nielsen, R. C. and Veech, R. L. (1973). The acute action of ammonia on rat brain metabolism in vivo. *Biochemical Journal*, **134**, 1001–1008.

[7] Hindfelt, B., Plum, F. and Duffy, T. E. (1977). Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. *Journal of Clinical Investigation*, **59**, 386–396.

[8] Kosenko, E., Kaminsky, Y., Felipo, V., Miñana, M. D. and Grisolía, S. (1993). Chronic hyperammonemia prevents changes in brain energy and ammonia metabolites induced by acute ammonia intoxication. *Biochimica et Biophysica Acta*, **1180**, 321–326.

[9] Kosenko, E., Kaminsky, Y., Grau, E., Miñana, M. D., Marcaida, G., Grisolía, S. and Felipo, V. (1994). Brain ATP depletion induced by acute ammonia intoxication in rats is mediated by activation of the NMDA receptor and Na^+,K^+-ATPase. *Journal of Neurochemistry*, **63**, 2172–2178.

[10] Kosenko, E., Felipo, V., Miñana, M. D., Grisolia, S. and Kaminsky, Y. (1997). Effects of acute hyperammonemia in vivo on oxidative metabolism in nonsynaptic rat brain mitochondria. *Metabolic Brain Disease*, **12**, 69–82.

[11] Marcaida, G., Miñana, M. D., Grisolía, S. and Felipo, V. (1995). Lack of correlation between glutamate-induced depletion of ATP and neuronal death in primary cultures of cerebellum. *Brain Research*, **695**, 146–150.

[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.

[13] Senior, A. E. (1979). Oligomycin-sensitivity-conferring protein. *Methods in Enzymology*, **LV**, 391–397.

[14] Kaminsky, Y. Senior, A. E. and Kosenko, E. (1988). Diurnal changes in succinate and D-3-hydroxybutyrate dehydrogenase activities of rat liver mitochondria after chronic alcohol consumption and withdrawal. *Comparative Biochemistry and Physiology C*, **90**, 79–82.

[15] Goldberg, D. M. and Spooner, R. J. (1984). Glutathione reductase. In *Methods of Enzymatic Analysis vol. 3* (ed. H. U. Bergmeyer), Wiley, New York, pp. 259–265.

[16] Lawrence, R. A. and Burk, R. F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and Biophysical Research Communications*, **71**, 952–958.

[17] Aebi, H. E. (1984). Catalase. In *Methods of Enzymatic Analysis vol. 3* (ed. H. U. Bergmeyer), Wiley, New York, pp. 273–286.

[18] Cohen, G., Dembiec, D. and Marcus, J. (1970). Measurement of catalase activity in tissue extracts. *Analytical Biochemistry*, **34**, 30–38.

[19] Beauchamp, C. and Fridovich, I. (1971). Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276–287.

[20] Boveris, A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods in Enzymology*, **105**, 429–435.

[21] Forman, H. J. and Kennedy, J. (1976). Dihidroorotate-dependent superoxide production in rat brain and liver. A function of the primary dehydrogenase. *Archives of Biochemistry and Biophysics*, **173**, 219–224.

[22] O'Connor, J. E. and Costell, M. (1990). New roles of carnitine metabolism in ammonia cytotoxicity. In *Cirrhosis, hepatic encephalopathy, and ammonium toxicity* (eds. S. Grisolía, V. Felipo and M. D. Miñana), Plenum Press, New York, pp. 183–195.

[23] Sanz, N., Diez-Fernández, C., Fernández-Simon, L., Alvarez, A. and Cascales, M. (1995). Relationship between antioxidant systems, intracellular thiols and DNA ploidy in liver of rats during experimental cirrhogenesis. *Carcinogenesis*, **16**, 1585–1593.

[24] Nadkarni, G. D. and D'souza, N. B. (1988). Hepatic antioxidant enzymes and lipid peroxidation in carbon tetrachloride-induced liver cirrhosis in rats. *Biochemical Medicine and Metabolic Biology*, **40**, 42–45.

[25] Corrocher, R., Casaril, M., Guidi, C., Gabrielli, G. B., Miatto, O. and De Sandre, G. (1980). Glutathione-peroxidase and glutathione-reductase activities of normal and pathologic human liver: relationship with age. *Scandinavian Journal of Gastroenterology*, **15**, 781–786.

[26] Ozenirler, S., Tuncer, C., Ongun, O., Altan, N. and Kandilci, U. (1994). Activities of superoxide dismutase in erythrocyte of nonalcoholic chronic liver diseases. *General Pharmacology*, **25**, 1349–1351.

[27] Loschen, G., Azzi, A., Richter, C. and Flohé, L. (1974). Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Letters*, **42**, 68–72.

[28] Sorgato, M. C., Sartorelli, L., Loschen, G. and Azzi, A. (1974). Oxygen radicals and hydrogen peroxide in rat brain mitochondria. *FEBS Letters*, **45**, 92–95.

[29] McCord, J. M. and Fridovich, I. (1969). Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049–6055.

[30] Miñana, M. D., Felipo, V. and Grisolía, S. (1988). Protective effect of long term ammonium ingestion against acute ammonium intoxication. *Biochemical and Biophysical Research Communications*, **153**, 979–983.

[31] Hermenegildo, C., Marcaida, G., Montoliu, C., Grisolia, S., Miñana, M. D. and Felipo, V. (1996). NMDA receptor antagonists prevent acute ammonia toxicity in mice. *Neurochemical Research*, **21**, 1237–1244.

[32] Felipo, V., Grau, E., Miñana, M. D. and Grisolía, S. (1993). Ammonium injection induces an *N*-methyl-D-aspartate receptor-mediated proteolysis of the microtubule-associated protein MAP-2. *Journal of Neurochemistry*, **60**, 1626–1630.

[33] Kosenko, E., Kaminsky, Y., Grau, E., Miñana, M. D., Grisolía, S. and Felipo, V. (1995). Nitroarginine, an inhibitor of nitric oxide synthetase, attenuates ammonia toxicity and ammonia-induced alterations in brain metabolism. *Neurochemical Research*, **20**, 451–456.

[34] Mohazzab, K. M., Fayngersh, R. P. and Wolin, M. S. (1996). Nitric oxide inhibits pulmonary artery catalase and H_2O_2-associated relaxation. *American Journal of Physiology*, **271**, H1900–H1906.

[35] Farias-Eisner, R., Chaudhuri, G., Aeberhard, E. and Fukuto, J. M. (1996). The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistence/susceptibility. *Journal of Biological Chemistry*, **271**, 6144–6151.

[36] Brown, G. C. (1995). Reversible binding and inhibition of catalase by nitric oxide. *European Journal of Biochemistry*, **232**, 188–191.

[37] Asahi, M., Fujii, J., Suzuki, K., Seo, H. G., Kuzuya, T., Hori, M., Tada, M., Fujii, S. and Taniguchi, N. (1995). Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. *Journal of Biological Chemistry*, **270**, 21035–21039.

[38] Bolanos, J. P., Peuchen, S., Heales, S. J., Land, J. M. and Clark, J. B. (1994). Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *Journal of Neurochemistry*, **63**, 910–916.

[39] Luperchio, S., Tamir, S. and Tannenbaum, S. R. (1996). NO-induced oxidative stress and glutathione metabolism in rodent and human cells. *Free Radicals in Biology and Medicine*, **21**, 513–519.

[40] Chou, S. M., Wang, H. S. and Taniguchi, A. (1996). Role of SOD-1 and nitric oxide/cyclic GMP cascade on neurofilament aggregation in ALS/MND. *Journal of Neurological Science*, **139 suppl**, 16–26.

[41] McCord, J. M. (1987). Oxygen-derived radicals: a link between reperfusion injury and inflammation. *Federation Proceedings*, **46**, 2402–2406.

[42] Thomas, G. and Ramwell, P. W. (1988). Vasodilatory properties of mono-L-arginine containing compounds. *Biochemical and Biophysical Research Communications*, **154**, 332–338.

[43] Wettstein, M., Gerok, W. and Häussinger, D. (1994). Endotoxin-induced nitric oxide synthesis in the perfused rat liver: effects of L-arginine and ammonium chloride. *Hepatology*, **19**, 641–647.