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Ammonia Neurotoxicity and the Mitochondrial Permeability Transition

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Ammonia is a neurotoxin that predominantly affects astrocytes. Disturbed mitochondrial function and oxidative stress, factors implicated in the induction of the mitochondrial permeability transition (MPT), appear to be involved in the mechanism of ammonia neurotoxicity. We have recently shown that ammonia induces the MPT in cultured astrocytes. To elucidate the mechanisms of the MPT, we examined the role of oxidative stress and glutamine, a byproduct of ammonia metabolism. The ammonia-induced MPT was blocked by antioxidants, suggesting a causal role of oxidative stress. Direct application of glutamine (4.5–7.0 mM) to cultured astrocytes increased free radical production and induced the MPT. Treatment of astrocytes with the mitochondrial glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine, completely blocked free radical formation and the MPT, suggesting that high ammonia concentrations in mitochondria resulting from glutamine hydrolysis may be responsible for the effects of glutamine. These studies suggest that oxidative stress and glutamine play major roles in the induction of the MPT associated with ammonia neurotoxicity.

KEY WORDS: Ammonia; astrocytes; glutamine; mitochondrial permeability transition; oxidative stress.

INTRODUCTION

Ammonia is a neurotoxin that has been strongly implicated in the pathogenesis of hepatic encephalopathy (HE), an important cause of morbidity and mortality in patients with severe liver failure. It is also an important factor in inborn errors of the urea cycle, Reye's syndrome, organic acidurias, valproate toxicity, transient hyperammonemia in infants, and idiopathic hyperammonemia.

The pathology of hyperammonemia, particularly HE, suggests that astrocytes play a crucial role in this condition (Norenberg, 1987). Astrocyte swelling represents the principal component of acute HE, while the presence of Alzheimer type II astrocytes is the main histological finding in chronic HE. No significant or consistent neuronal changes have been identified (Norenberg, 1981). Because of the critical role of astrocytes in neurotransmission and CNS bioenergetics, we have proposed that astroglial dysfunction (gliopathy) and associated derangement in glial–neuronal interactions represent major aspects in the pathogenesis of ammonia neurotoxicity (Norenberg *et al*., 1997).

Cerebral ammonia is chiefly metabolized to glutamine in astrocytes, due to predominant localization of glutamine synthetase in these cells (Norenberg and Martinez-Hernandez, 1979). Physiological levels of glutamine thus formed in astrocytes is released into the extracellular space and is taken up by neurons to generate glutmate and ammonia, a reaction mediated by phosphate-activated glutaminase (PAG). In addition, glutamine can also be metabolized to glutamate and ammonia in astrocytes, as evidenced by studies in culture (Kvamme *et al*., 1992) as well as in vivo (Subbalakshmi and Murthy, 1985) showing that astrocytes possess PAG.

This article highlights the role of the mitochondrial permeability transition (MPT) as a major factor in the cellular dysfunction associated with ammonia neurotoxicity. The role of oxidative stress will be emphasized as a

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causal factor in the induction of the MPT. Mitochondrial dysfunction resulting from ammonia neurotoxicity as a consequence of the MPT will be discussed. Lastly, recent concepts on potential mechanism(s) of the ammonia-induced MPT will be presented.

CEREBRAL ENERGY METABOLIC FAILURE IN AMMONIA TOXICITY

The concept that ammonia disturbs cerebral energy metabolism has long been proposed (see Rama Rao and Norenberg, 2001 and references therein). Ammonia is known to interfere with various metabolic pathways of cerebral energy metabolism including inhibition of α-ketoglutarate dehydrogenase; stimulation of Na^+, K^+-ATPase resulting in depletion of ATP; impairment in the oxidation of pyruvate and glutamate; disturbance in the operation of the malate-aspartate shuttle; reduction in the state III mitochondrial respiration; and inhibition of the activity and expression of electron transport chain enzymes. Some of the abnormalities have been reproduced in cultured astrocytes exposed to pathophysiological concentrations of ammonia. In addition, several studies have shown morphologic changes in mitochondria in HE/hyperammonemia, principally swelling of the matrix and intracristal space (Gregorios *et al*., 1985; Norenberg, 1977; Norenberg *et al*., 2002).

OXIDATIVE STRESS IN AMMONIA TOXICITY

Oxidative stress is an evolving concept in HE and ammonia toxicity. Increased superoxide production and reduced activities of antioxidant enzymes have been reported in brains of rats subjected to acute ammonia toxicity (Kosenko *et al*., 1997). Consistent with these findings, biphasic responses of total glutathione (GSH) were identified in cultured astrocytes exposed to 5 mM NH_4Cl. At early time points (up to 6 h) GSH levels were reduced by ammonia, whereas at later time points (up to 72 h), a progressive increase in GSH content occurred (Murthy *et al*., 2000a,b). Lowered levels of GSH in astrocytes in early phase of ammonia exposure is consistent with the concept that ammonia induces oxidative stress in astrocytes. The later increase in GSH may represent an adaptive response to oxidative stress.

To examine the cellular basis of oxidative stress in ammonia toxicity, free radical production was measured employing the fluorescent probe 5-(and-6)carboxy-2′-7′-dichlorofluorescein diacetate (DCFDA). These studies demonstrated that ammonia stimulated the production of free radicals in a dose-dependent manner. These data also disclosed that ROS levels remained elevated for at least 4 h after exposure to ammonia. At the earliest time point (3 min) there was a robust increase in free radical production followed by a transient but significant reduction up to 2 h (but still higher than control); at 4 h the increase was similar to that observed at the 3 min time point (Murthy *et al*., 2001; Rama Rao *et al*., 2003a). This pattern of increase in ROS production by ammonia (2–4 h) is consistent with a concomitant decrease (up to 6 h) in astrocytic GSH levels as described above.

THE MITOCHONDRIAL PERMEABILITY TRANSITION

The potential involvement of mitochondrial dysfunction and oxidative stress in ammonia neurotoxicity prompted our investigation into the possible role of the mitochondrial permeability transition (MPT) in hyperammonemia. The MPT is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to small molecules ($<$1500 Da). This is due to the opening of a specific permeability transition pore in the inner mitochondrial membrane, usually in response to an increase in mitochondrial Ca^{2+} levels. This leads to a collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) that is created by the pumping out of protons by the electron transport chain. Loss of the $\Delta\Psi_m$ leads to colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and the generation of ROS. For reviews, see Zoratti and Szabo (1995) and Bernardi *et al*. (1998). The most specific blocker of the MPT is cyclosporin A (CsA), which competitively inhibits the mitochondrial matrix protein cyclophilin D from binding to pore domains (Crompton *et al*., 1998).

To determine whether ammonia treatment of cultured astrocytes was associated with a change in the $\Delta\Psi_m$, a consequence of the MPT, astrocytes were treated with 5 mM NH_4Cl and examined for changes in the $\Delta\Psi_m$ using the potentiometric fluorescent dyes JC-1 and TMRE. Astrocytes exposed to ammonia showed a significant dissipation of the $\Delta\Psi_m$ in a time-and concentration-dependent manner. These studies also demonstrated that pretreatment with CsA (1–5 μM) blocked the ammonia-induced dissipation of the $\Delta\Psi_m$ (Bai *et al*., 2001; Rama Rao *et al*., 2003a) (Fig. 1), suggesting that ammonia was inducing the MPT.

To directly visualize permeability changes in mitochondria in situ, the calcein fluorescence method was employed (Petronilli *et al*., 1999). Calcein/AM enters cells and becomes fluorescent upon de-esterification. Coloading of cells with cobalt chloride quenches the fluorescence in the cell, except in mitochondria, since cobalt is

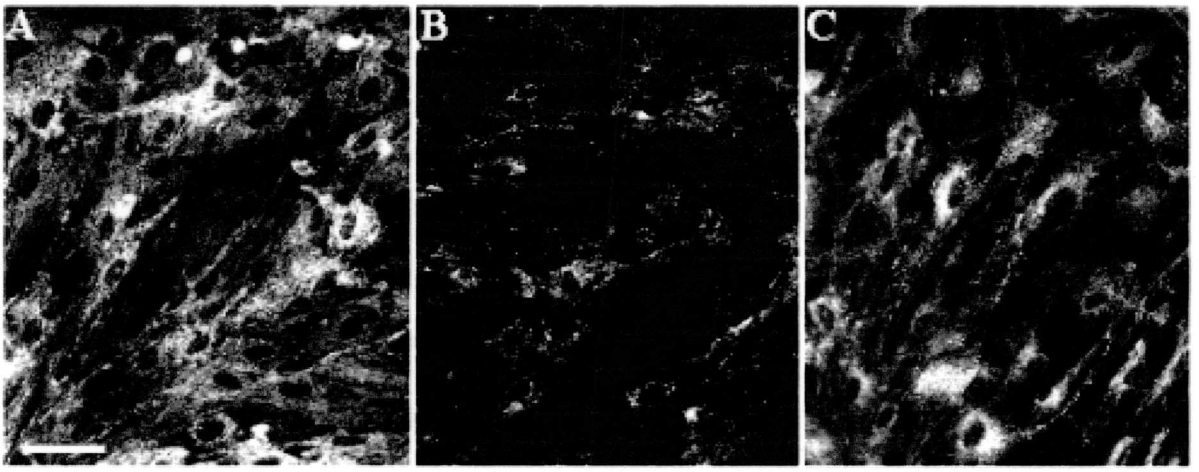

Fig. 1. Effect of 5 mM NH_4Cl on TMRE fluorescence in cultured astrocytes. Cells were loaded with 25 nM TMRE for 20 min. (A) Control astrocytes show prominent fluorescence. (B) Ammonia-treated astrocytes show decreased fluorescence. (C) Astrocytes treated with 1 μM CsA and ammonia is similar to control. Scale bar, 10 μm.

impermeable across mitochondrial membranes. However, during induction of the MPT, cobalt enters mitochondria and quenches the calcein fluorescence. Treatment of cultured astrocytes with ammonia (24 h) caused a significant reduction in the fluorescent intensity of calcein, which was significantly blocked by pretreatment with CsA (1 μM) (Fig. 2).

The ammonia-induced MPT in cultured astrocytes was significantly attenuated by various antioxidants, including SOD (25 U/mL), catalase (250 U/mL), desferroxamine (40 μM), *N*-*t*-butyl-α-phenyl-nitrone (PBN; 250 μM), supporting the notion that oxidative stress plays a major role in the ammonia-induced MPT in astrocytes (Jayakumar *et al*., 2002).

ROLE OF GLUTAMINE IN THE MECHANISM OF AMMONIA NEUROTOXICITY

While ammonia is believed to be responsible for the neurological abnormalities associated with HE and other hyperammonemic syndromes, growing evidence

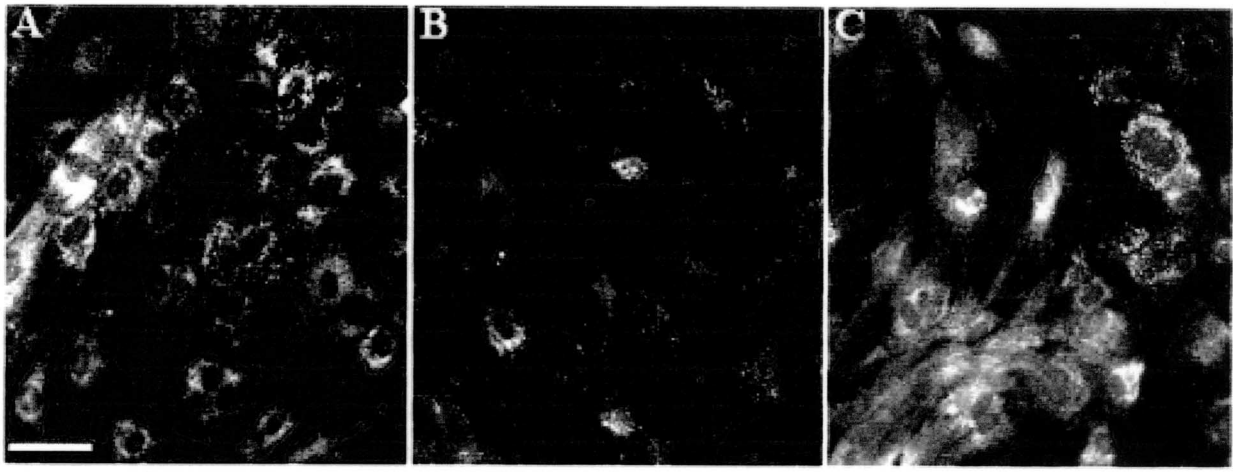

Fig. 2. Induction of the MPT in astrocytes by ammonia as demonstrated by calcein fluorescence. (A) Control astrocytes loaded with 1 μM calcein and quenched with cobalt show brightly stained mitochondria. (B) Astrocytes treated with ammonia (5 mM) for 24 h and then loaded with calcein show a significant loss of mitochondrial calcein fluorescence, consistent with the induction of the MPT. (C) Cotreatment with CsA (1 μM) prevents the loss of calcein fluorescence by ammonia. Scale bar, 10 μm.

supports the view that glutamine, a byproduct of ammonia metabolism, plays a major role in the deleterious effects of ammonia. Various abnormalities associated with ammonia toxicity such as seizures, depressed glucose utilization, altered CNS metabolism, vascular CO_2 responsiveness, edema, and astrocyte swelling can be blocked by administration of methionine sulfoxamine (MSO), an inhibitor of glutamine synthetase (Rama Rao *et al*., 2003b and references therein).

Earlier studies showed that MSO completely blocked the effect of ammonia on the MPT (Bai *et al*., 2001), as well as free radical production (Murthy *et al*., 2001). These findings suggested that glutamine was mediating the effects of ammonia on the MPT and free radical formation. Subsequent studies have examined the role of glutamine directly. Cultured astrocytes treated with glutamine (4.5–7 mM for 24 h) caused a significant dissipation of $\Delta\Psi_m$ as well as decreased mitochondrial calcein fluorescence, both of which were completely blocked by CsA (Rama Rao *et al*., 2003b). In addition, glutamine significantly increased free radical production in cultured astrocytes, which was also completely blocked by CsA (Jayakumar *et al*., 2004).

To investigate the potential mechanism by which glutamine induces free radicals and the MPT, cultured astrocytes were treated with 6-diazo-5-oxo-L-norleucine (DON; 1 mM), an inhibitor of phosphate-activated glutaminase (PAG). DON completely blocked the glutamine-induced free radical production (Jayakumar *et al*., 2004). Since essentially all of the glutamine is metabolized in mitochondria by PAG, high levels of ammonia will be generated in these organelles leading to the production of free radicals and the induction of the MPT. We envision glutamine acting as a "Trojan horse" by providing high levels of ammonia, leading to oxidative stress and mitochondrial dysfunction.

ASTROCYTIC MITOCHONDRIA ARE MORE VULNERABLE TO THE AMMONIA-INDUCED MPT

It is noteworthy that astrocytic rather than neuronal mitochondria are predominantly vulnerable to MPT induction by ammonia (Bai *et al*., 2001). Similarly, glutamine had no effect on free radical production in cultured neurons (Jayakumar *et al*., 2004). There are two possibilities to explain these findings. First, there is evidence of heterogeneity of mitochondria among neurons and astrocytes (Blokhuis and Veldstra, 1970), and it is possible that neuronal mitochondria may be more resistant to induction of the MPT by ammonia. Supporting this possibility, Fiskum *et al*. (2000) demonstrated a greater resistance of neuronal mitochondria to the effects of Ca^{2+} overload and the subsequent induction of the MPT as compared with astrocytic mitochondria. Second, the selective vulnerability of astrocytes to the ammonia-induced MPT may be due to high levels of glutamine in astrocytes since ammonia is metabolized to glutamine in astrocytes but not in neurons.

ROLE OF THE MPT IN ASTROCYTE SWELLING

Astrocyte swelling represents a significant component of the brain edema in fulminant hepatic failure (FHF) (Córdoba and Blei, 1996). While the mechanism of edema associated with FHF is not completely understood, elevated ammonia levels have been strongly implicated in this disorder (Clemmesen *et al*., 1999). Studies employing cultured astrocytes (Norenberg *et al*., 1991) and brain slices (Ganz *et al*., 1989) exposed to pathophysiological concentrations of ammonia have demonstrated prominent astrocyte swelling. More recently, ammonia has been shown to upregulate the water channel protein aquaporin4 (AQP4), suggesting that AQP4 may be responsible for astrocyte swelling (Rama Rao and Norenberg, 2003c). Collectively, there is compelling evidence that supports a major role of ammonia in the astrocyte swelling associated with hyperammonemia.

Since ammonia has been shown to induce the MPT and mitochondrial dysfunction, the role of the MPT on astrocyte swelling was assessed. Pretreatment of cultured astrocytes with different concentrations of CsA (0.1–1 μM) significantly blocked the astrocyte swelling caused by ammonia. Parallel studies also demonstrated that CsA treatment significantly blocked the ammonia-mediated increase in AQP4 expression (Rama Rao and Norenberg, 2003d). Additionally, antioxidants significantly blocked the ammonia-induced astrocyte swelling (Murthy *et al*., 2000). These studies support the role of the MPT and oxidative stress in the astrocyte swelling and brain edema associated with hyperammonemic states.

CONCLUDING REMARKS

In summary, ammonia induces the MPT in cultured astrocytes but not in cultured neurons, highlighting the critical role that astrocytes play in the toxic effects of ammonia. These effects of ammonia on the MPT were prevented by cyclosporin A. Ammonia-induced astrocyte swelling was blocked by CsA suggesting a major role of the MPT in this process. Our studies also suggest that

glutamine likely mediates the effect of ammonia in the induction of oxidative stress as well as the MPT. We propose that oxidative stress and the MPT represent key pathogenetic factors in ammonia neurotoxicity. These findings provide potential therapeutic targets for HE and other hyperammonemic states.

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REFERENCES

Bai, G., Rama Rao, K. V., Murthy, Ch. R. K., Panickar, K. S., Jayakumar, A. R., and Norenberg, M. D. (2001). *J. Neurosci. Res.* **66**, 981–991.

Bernardi, P., Colonna, R., Costantini, P., Eriksson, O., Fontaine, E., Ichas, F., Massari S., Nicolli, A., Petronilli, V., and Scorrano, L. (1998). *Biofactors* **8**, 273–281.

Blokhuis, G. G. D., and Veldstra, H. (1970). *FEBS Lett.* **11**, 197–199.

Clemmesen, J. O., Larsen, F. S., Kondrup, J., Hansen, B. A., and Ott, P. (1999). *Hepatology* **19**, 1437–1444.

Córdoba, J., and Blei, A. T. (1996). *Semin. Liver. Dis.* **16**, 271–280.

Crompton, M., Virji, S., and Ward, J. M. (1998). *Eur. J. Biochem.* **258**, 729–735.

Fiskum, G., Chandrasekharan, K., Mehrabyan, Z., Krueger, B. K., and Bambrick, L. L. (2000). *J. Neurochem.* (Suppl.), S95A.

Ganz, R., Swain, M., Traber, P., Dal Canto, M. C., Butterworth, R. F., and Blei, A. T. (1989). *Metab. Brain Dis.* **4**, 213–223.

Gregoris, J. B., Mozes, L. W., and Norenberg, M. D. (1985). J. Neuropathol. *Exp. Neurol.* **44**, 404–414.

Jayakumar, A. R., Rama Rao, K. V., and Norenberg, M. D. (2002). Role of oxidative stress in the ammonia-induced mitochondrial permeability transition in cultured astrocytes. *J. Neurochem.* **81**(Suppl), 109.

Jayakumar, A. R., Rama Rao, K. V., and Norenberg, M. D. (2004). Glutamine-induced free radical production in cultured astrocytes. *Glia*. **46**, 296–301.

Kosenko, E., Kaminsky, M., Kaminsky, A., Valencia, M., Lee, L., Hermenegildo, C., and Felipo, V. (1997). *Free Radic. Res.* **27**, 637–644.

Kvamme, E., Svenneby, G., Hertz, L., and Schousboe, A. (1982). *Neurochem. Res.* **7**, 760–767.

Murthy, C. R., Bai, G., Dombro, R. S., and Norenberg, M. D. (2000a). *Soc. Neurosci. Abstr.* **26**, 1893.

Murthy, Ch. R. K., Bender, A. S., Dombro, R. S., Bai, G., and Norenberg, M. D. (2000b). *Neurochem. Int.* **37**, 255–268.

Murthy, Ch. R. K., Rama Rao, K. V., Bai, G., and Norenberg, M. D. (2001). *J. Neurosci. Res.* **66**, 282–288.

Norenberg, M. D. (1977). *Lab. Invest.* **36**, 618–627.

Norenberg, M. D. (1981). In *Advances in Cellular Neurobiology*, Vol. 2 (Fedoroff, S., and Hertz, L., eds.), Academic Press, New York, pp. 303–352.

Norenberg, M. D. (1987). *Neurochem. Pathol.* **6**, 13–33.

Norenberg, M. D., Baker, L., Norenberg, L.-O. B., Blicharska, J., Bruce-Gregorios, J. H., and Neary, J. T. (1991). *Neurochem. Res.* **16**, 833–836.

Norenberg, M. D., Huo, Z., Neary, J. T., and Roig-Cantesano, A. (1997). *Glia* **21**, 124–133.

Norenberg, M. D., and Martinez-Hernandez, A. (1979). *Brain Res.* **161**, 303–310.

Norenberg, M. D., Rama Rao, K. V., and Jayakumar, A. R. (2002). In *Hepatic Encephalopathy and Nitrogen Metabolism* (Jones, E. A., Meijer, A. F., and Chamuleau, R. A. eds.), Kluwer, Dordtecht, The Netherlands, pp. 267–287

Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P., and Di Lisa, M. (1999). *Biophys. J.* **765**, 725–754.

Rama Rao, K. V., Chen, M., Simard, J. M., and Norenberg, M. D. (2003d). *J. Neurosci. Res.* **74**, 891–897.

Rama Rao, K. V., Chen, M., Simard, J. M., and Norenberg, M. D. (2003c). *Neuroreport* **14**, 2379–2382.

Rama Rao, K. V., Jayakumar, A. R., and Norenberg, M. D. (2003a). *Metab. Brain Dis.* **18**, 113–127.

Rama Rao, K. V., Jayakumar, A. R., and Norenberg, M. D. (2003b). *Neurochem. Int.* **43**, 517–524.

Rama Rao, K. V., and Norenberg, M. D. (2001). *Metab. Brain Dis.* **16**, 67–78.

Subbalakshmi, G. Y., and Murthy, Ch. R. K. (1985). *Neurochem. Res.* **10**, 239–250.

Zoratti, M., and Szabo, I. (1995). *Biochim. Biophys. Acta* **1241**, 139–176.