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Review

Oxidative and nitrosative stress in ammonia neurotoxicity

Marta Skowrońska, Jan Albrecht *

Department of Neurotoxicology, Mossakowski Medical Research Center, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland

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ABSTRACT

Increased ammonia accumulation in the brain due to liver dysfunction is a major contributor to the pathogenesis of hepatic encephalopathy (HE). Fatal outcome of rapidly progressing (acute) HE is mainly related to cytotoxic brain edema associated with astrocytic swelling. An increase of brain ammonia in experimental animals or treatment of cultured astrocytes with ammonia generates reactive oxygen and nitrogen species in the target tissues, leading to oxidative/nitrosative stress (ONS). In cultured astrocytes, ammonia-induced ONS is invariably associated with the increase of the astrocytic cell volume. Interrelated mechanisms underlying this response include increased nitric oxide (NO) synthesis which is partly coupled to the activation of NMDA receptors and increased generation of reactive oxygen species by NADPH oxidase. ONS and astrocytic swelling are further augmented by excessive synthesis of glutamine (Gln) which impairs mitochondrial function following its accumulation in there and degradation back to ammonia ("the Trojan horse" hypothesis). Ammonia also induces ONS in other cell types of the CNS: neurons, microglia and the brain capillary endothelial cells (BCEC). ONS in microglia contributes to the central inflammatory response, while its metabolic and pathophysiological consequences in the BCEC evolve to the vasogenic brain edema associated with HE. Ammonia-induced ONS results in the oxidation of mRNA and nitration/nitrosylation of proteins which impact intracellular metabolism and potentiate the neurotoxic effects. Simultaneously, ammonia facilitates the antioxidant response of the brain, by activating astrocytic transport and export of glutathione, in this way increasing the availability of precursors of neuronal glutathione synthesis.

1. Introduction

Ammonia is a neurotoxin implicated in neurological disorders associated with hyperammonemia (HA) (Felipo and Butterworth, 2002). Among these, hepatic encephalopathy (HE), where excessive accumulation of ammonia results from its impaired detoxification due to liver failure is the major epidemiological burden; in EU countries, the death rate associated with liver cirrhosis exceeds that associated with diabetes or car accidents (Eurostat: http://ec.europa.eu/health/ph_information).

HE is a complex neurological disorder manifested by a variety of pathophysiological symptoms and biochemical manifestations, which have been subject to many exhaustive reviews (Albrecht and Jones, 1999; Butterworth, 2010; Córdoba, 2011; Häussinger, 2010). HE is a dynamic and progressive disease, which with regard to escalation of neuropsychiatric symptoms may progress from grade 0 (in which no overt abnormalities are detected) to grade IV, which refers to patients in coma, unresponsive to external stimuli (Conn et al., 1977; Weissenborn et al., 2001; Ferenci et al., 2002). Mental and motor abnormalities in HE evolve from slightly impaired behavior (lack of awareness, euphoria or anxiety), through lethargy, disorientation and somnolence to confusion, gross disorientation and finally coma (Bajaj et al., 2011). Of note in the context of the present review, HE differs from typically neurodegenerative disorders of the CNS in that: (i) its major manifestations are either spontaneously reversible or can be reversed by correcting liver function, and (ii) neuronal dysfunction is largely secondary to astrocytic impairment. There is a consensus that brain edema, which is the major cause of death in rapidly progressing ("acute") HE, is primarily due to astrocytic swelling (reviewed in Norenberg, 1996; Vaquero et al., 2003).

The vulnerability of astrocytes in the setting of HE has already been documented by the pioneering light and electron microscopic studies carried out a few decades ago. Astrocytes in humans and different HE models present, among other changes, cytoplasmic enlargement, degenerative mitochondrial changes and enlarged nuclei, in its extreme form assuming the appearance of the Alzheimer type II astrocytes (Norenberg and Lapham, 1974; Diemer, 1977; Norenberg, 1977) and swelling of perivascular astrocytic foot-processes (Pilbeam et al., 1983).

At the brain biochemical level, the mechanisms underlying HE include a wide range of metabolic derangements contributed not only by ammonia, but also by other toxins derived from the periphery. Indeed, recent evidence indicates that inflammation

* Corresponding author. Tel.: +48 22 6086617; fax: +48 22 60886442.
E-mail addresses: mskowronska@cmdik.pan.pl (M. Skowrońska), jalbrecht@imdik.pan.pl (J. Albrecht).

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and infection synergistically enhance the deleterious effects of ammonia, including ONS (Shawcross et al., 2010). However, the text below focuses on biochemical events directly related to the action of ammonia in the CNS. Model studies carried out until now have largely dealt with this pathogen, and to a much lesser degree with the effects of low-grade inflammation. Logically, we owe the recognition of the role of ONS in the pathogenesis of HE to studies examining the effect of ammonia.

Disturbances of energy metabolism arising from mitochondrial dysfunction have long been considered as a key pathogenic factor in neurodegenerative diseases. As outlined by other contributors to the present issue, ONS is a major consequence of mitochondrial dysfunction and a ubiquitous contributor to the pathogenesis of neurodegenerative disorders. By contrast, early evidence regarding the role of disturbances of energy metabolism in HE has been ambiguous. In the earlier studies, contrasting results could be ascribed to the different models used but also to the lack of experimental tools to analyze the distinct responses of astrocytes vs. other cellular components of the CNS. Whole brain glucose consumption was found reduced in some HE models, like the portacaval shunt (PCS) model (DeJoseph and Hawkins, 1991) or thioacetamide-induced liver failure (Pluta and Albrecht, 1986; Hilgier et al., 1991). By contrast, Hindfelt and colleagues using an acute-upon-chronic HE model (PCS + ammonia bolus) observed an increase of accumulation in the brain of glycolytic intermediates, paralleled by enhanced lactate/pyruvate ratio, with simultaneous decline of citrate and ATP, apparently manifesting impaired conversion of pyruvate to citrate (Hindfelt et al., 1977). However, in one other study using a straight PCS model, no increase of the metabolic rate for glucose was observed in most of the brain regions (Lockwood et al., 1986). In a study accounting simultaneously for the response to HE of the different CNS cell types, a 30% decrease of oxygen consumption was noted in astrocytes, while at the same stage the oxygen consumption in neurons was increased by some 35% (Albrecht et al., 1987). These results rendered further support to the view that astrocytes are the cells primarily damaged during hepatic encephalopathy and that HE is primarily a disease of astrocytes.

The concept that astrocytes may be a specific target of ammonia neurotoxicity was born in the 1970s, with the seminal observation that glutamine synthetase (GS), the enzyme converting glutamate and ammonia to glutamine, is located primarily, if not exclusively, in astrocytes (Martinez-Hernandez et al., 1977). Considering the incompleteness of the urea cycle, glutamine synthesis is the only efficient route of ammonia neutralization in the brain (Cooper and Plum, 1987). However, as will be outlined in the following sections, in HE, excessive accumulation of ammonia-derived Gln contributes to ammonia-induced ONS, perhaps no less than ammonia itself. Changes that involve defined steps in brain metabolism are often interrelated with biophysical changes, such as alterations in intracellular pH. For instance, alkalinization of glial pH in hyperammonemic rats (from the normal baseline value of 7.1 to about 7.4–7.5) was found to correlate with brain ammonia concentration and severity of encephalopathy (Kanamori and Ross, 1997).

These metabolic and biophysical changes conjunctively contribute to ammonia-induced ONS in astrocytes. However, as outlined in the forthcoming sections, HE evokes ONS in neurons, microglia and the blood–brain barrier-forming endothelial cells as well.

2. Mechanisms by which ammonia induces ONS in astrocytes

2.1. Direct routes

ONS induces a cascade of pathogenic events including pathophysiologic changes in astrocytes, perturbation in astrocyte–neuron crosstalk and synaptic plasticity disorders, which underlie different symptoms of HE. Generation of reactive oxygen and nitrogen species (ROS/RNS) including the highly toxic peroxynitrite ($ONOO^-$), has been most frequently reported in experiments using ammonia-treated astrocytes in culture (Murthy et al., 2001; Görg et al., 2008; Skowrońska et al., 2010). Increased production of the hydroxyl radicals (·OH) was also observed in brain *in vivo*, in a model in which ammonium chloride was added directly to the striatum through microdialysis (Hilgier et al., 2003). Hyperammonemia *in vivo*, as well as treatment of astrocytes with 5–10 mM ammonia, which is thought to mimic the critical hyperammonemia-induced changes in these cells, are associated with increased expression and activity of heme oxygenase-1 (HO-1), a ubiquitous marker of oxidative stress (Warskulat et al., 2002; Rama Rao et al., 2010). Increased activity and expression of nitric oxide synthases (NOS) (Rao et al., 1995) and increased levels of nitrites and nitrates (markers of NO production) were also detected in the brains of animals with experimentally induced HE (Genesca et al., 1999; Master et al., 1999). On the other hand, HA was also found to be associated with a decreased activity of antioxidant enzymes (glutathione peroxidase, superoxide dismutase and catalase) in the brain, both in the cytosolic and mitochondrial fractions (Kosenko et al., 1997). Of note, manipulations resulting in the recovery of some enzyme activities ameliorated HE symptoms both in experimental animals (Jiang et al., 2009b), and in patients (Sushma et al., 1992).

Studies with cultured astrocytes disclosed two, mutually not exclusive mechanisms of ONS generation by ammonia. The first one consists in over-stimulation of NMDA receptors leading to increased Ca^{2+}-dependent intracellular responses. The presence of active NMDA receptors in astrocytes has for years been a matter of controversy: attempts to demonstrate them in cultured cells have often been futile (Pearce et al., 1986). However, the controversy may have been technical in nature, since functional NMDA receptors have eventually been demonstrated in cultured astrocytes (Görg et al., 2003; Benz et al., 2004) and *in vivo* (Schipke et al., 2001). Accordingly, the NMDA receptor antagonist MK-801 blocked ammonia-induced generation of ROS/RNS in astrocytes (Kruczek et al., 2011), and the downstream signaling pathways, including phosphorylation of tyrosine residues on MAP-kinases Erk1/Erk-2 and p38MAPK (Schliess et al., 2002). In this light, the consequences of NMDA receptor-mediated increase of intracellular Ca^{2+} recorded in the ammonia-exposed brain: activation of the constitutive form of NO synthase, with subsequent formation of RNS (for references see Cagnon and Braissant, 2007), are likely to involve astrocytic response. It is tempting to speculate that *in situ*, activation of astrocytic NMDA receptors by ammonia is facilitated by increased Glu availability at the receptor site. Firstly, HA and HE, induced experimentally in various animal models, are often accompanied by increased extracellular Glu (Moroni et al., 1983; de Knegt et al., 1994; Hilgier et al., 1999). Secondly, ammonia induces Glu release from cultured astrocytes (Rose et al., 2005; Ohara et al., 2009), which tentatively may lead to self-amplifying autocrine activation of astrocytic NMDA receptors.

Involvement of NMDA receptors in ONS induction by ammonia has also been demonstrated *in vivo*. Acute intoxication of mice with large ammonia doses was associated with over-activation of NMDA receptors (Marcaida et al., 1992; Hermenegildo et al., 1996). The death rate of the animals, and the accompanying increase of the accumulation of superoxide in submitochondrial particles isolated *ex vivo*, and the reduced activities of antioxidant defense enzymes: superoxide dismutase (SOD), catalase and glutathione peroxidase, all were attenuated by NMDA receptor antagonists (Kosenko et al., 1999, 2003a). Acute ammonia intoxication of mice in the same paradigm (Hermenegildo et al., 2000), or intracerebral administration of ammonia to rats (Hilgier et al., 2003), both

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