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ORIGINAL PAPER

Evidence for oxidative/nitrosative stress in the pathogenesis of hepatic encephalopathy

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Abstract Hepatic encephalopathy (HE) is a serious complication of liver failure. HE manifests as a series of neuropsychiatric and neuromuscular symptoms including personality changes, sleep abnormalities, asterixis and muscle rigidity progressing through stupor to coma. The pathophysiologic basis of HE remains unclear. There is general agreement that ammonia plays a key role. In recent years, it has been suggested that oxidative/nitrosative stress constitutes part of the pathophysiologic cascade in HE. Direct evidence for oxidative/nitrosative stress in the pathogenesis of HE has been demonstrated in experimental animal models of acute or chronic liver failure. However, evidence from studies in HE patients is limited. This review summarizes this evidence for a role of oxidative/nitrosative stress in relation to ammonia toxicity and to the pathogenesis of HE.

Keywords Hepatic encephalopathy · Oxidative stress · Antioxidants · Liver failure

Abbreviations

HE	hepatic encephalopathy
ALF	acute liver failure
eNOS	endothelial nitric oxide synthase
HO-1	heme oxygenase-1
TAA	thioacetamide
SOD	superoxide dismutase
MPT	mitochondrial permeability transition
PTP	permeability transition pore
NAC	n-acetylcysteine
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NMDAr	n-methyl-d-aspartic acid receptors
GS	glutamine synthetase
MSO	methionine sulfoximine

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Introduction

Hepatic encephalopathy (HE) manifests as a series of neuropsychiatric and neuromuscular symptoms and is a serious complication of liver failure. The pathophysiologic basis of HE remains unclear. However, there is general agreement that ammonia plays a key role (Butterworth 2002). Ammonia accumulates in the brain in both acute and chronic liver failure leading to impaired bioenergetics (Kosenko et al. 1994; Rao and Norenberg 2001), altered neurotransmission (Mousseau and Butterworth 1994; Norenberg et al. 1997; Szerb and Butterworth 1992), activation of peripheral benzodiazepine receptors (Desjardins and Butterworth 2002; Norenberg et al. 1997) leading to the synthesis of neurosteroids (Ahboucha et al. 2006) as well as glutamate-mediated excitotoxicity (Hermenegildo et al. 1996; Marcaida et al. 1992) and excessive production of glutamine (Hawkins et al. 1993; Takahashi et al. 1991).

In recent years, oxidative/nitrosative stress has also been suggested to be part of the pathophysiologic cascade in HE. Oxidative stress is a condition in which the production of free radicals is far in excess of their rate of detoxification by endogenous mechanisms (Rao 2002). The brain is abso-

lutely dependent upon oxidative metabolism for cell survival and, being a highly aerobic tissue accounting for 20% of total oxygen consumed by the body, is prone to dysfunction due to oxidative stress.

More than a decade ago, O'Connor and Costell (1990) were among the first to postulate that oxidative stress is implicated in the pathophysiologic mechanisms responsible for HE. They reported that hyperammonemic mice displayed evidence of lipid peroxidation in the brain. Since then, evidence for a role of oxidative stress due to ammonia neurotoxicity has been confirmed from cell culture studies (Norenberg et al. 2007; Murthy et al. 2001; Rama Rao et al. 2003). However, direct evidence for oxidative/nitrosative stress in the pathogenesis of HE in either experimental animal models of liver failure or in material from patients is limited.

This short review will summarize evidence for a role of oxidative/nitrosative stress in relation to the pathogenesis of HE.

Acute liver failure

Several studies described evidence in support of a role of oxidative stress in HE due to acute liver failure (ALF). Selective increases in endothelial nitric oxide synthase (eNOS) mRNA expression in the frontal cortex of rats with ischemic liver failure were described (Sawara et al. 2009) as well as increased nitrite/nitrate and nitric oxide levels in these animals (Jiang et al. 2009a, b) and in other models of ALF (Sathyasaikumar et al. 2007; Master et al. 1999). Increased brain nitric oxide production was also reported in brain tissue from portacaval-shunted rats administered ammonia infusions to precipitate brain edema (Master et al. 1999). Heme oxygenase-1 (HO-1) mRNA expression was shown to be elevated in brain in experimental ALF due to azoxymethane administration (Fig. 1) (Bemeur et al. 2009) as well as in hepatic devascularized rats (Jiang et al. 2009a, b).

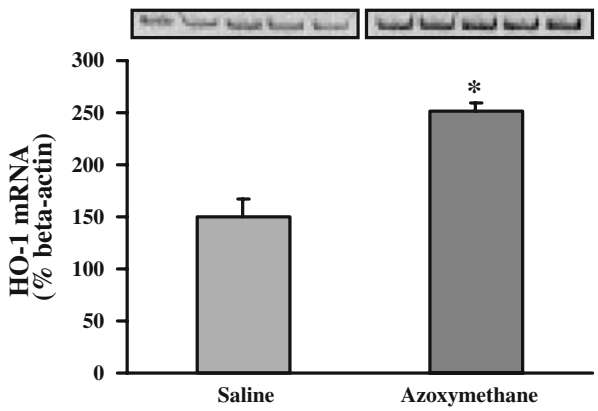

Fig. 1 Upregulation of brain HO-1 mRNA expression in the frontal cortex of azoxymethane-induced ALF mice compared to saline control mice. Data represent mean ± S.E.M. of $n=5$ animals per experimental group. * $p<0.001$ vs Saline

Catalase and glutathione peroxidase activities are decreased in the brains of rats with ALF due to thioacetamide (TAA) administration (Túnez et al. 2007; Sathyasaikumar et al. 2007). This loss of antioxidant defenses was accompanied by a higher rate of lipid peroxidation (Túnez et al. 2007; Sathyasaikumar et al. 2007), an increase of superoxide-dismutase (SOD)-induced hydrogen peroxide production (Reddy et al. 2004) and increased vulnerability to reactive oxygen species due to iron accumulation (Halliwell 1992; Sathyasaikumar et al. 2007). It was recently demonstrated that the total antioxidant status was significantly decreased in the rat brain following TAA-induced ALF (Zarros et al. 2008).

Mitochondrial permeability transition (MPT) is another potential consequence of oxidative stress. MPT is a Ca^{2+}-dependent mechanism associated with a collapse of the inner mitochondrial membrane potential due to a sudden opening of the permeability transition pore (PTP). Opening of the PTP results in enhanced free radical production (Miller et al. 2003). Ammonia has been shown to induce the MPT in cultured astrocytes (Bai et al. 2001), and the mechanism most likely involves oxidative stress as antioxidants completely inhibit its formation (Rama Rao et al. 2005). However, *in vivo* evidence of the involvement of MPT in HE awaits future studies.

Indomethacin, a nonsteroidal anti-inflammatory drug, has been reported to be beneficial in experimental ALF (Chung et al. 2001). Indomethacin is a non-selective cyclooxygenase inhibitor and thus would be expected to reduce the extent of oxidative stress by altering the arachidonate cascade. In addition, Asanuma et al. (2001) reported that indomethacin was able to scavenge nitric oxide radicals. In a single case report, indomethacin was shown to normalize the intracranial pressure in a patient with ALF (Clemmesen et al. 1997).

Our group recently demonstrated that the antioxidant N-acetylcysteine (NAC) significantly delayed the progression of encephalopathy in an experimental model of azoxymethane-induced ALF in the mouse (Bemeur et al. 2010a). NAC treatment decreased brain water content and proinflammatory cytokine levels and concomitantly restored both liver and brain GSH/GSSG ratios (Bemeur et al. 2010a). Treatment of experimental animals with HE with a range of agents with antioxidant properties including ascorbate, α-tocopherol, deferoxamine, butylated-hydroxyanisole, melatonin, L-carnitine, dimethylsulfoxide, and dimethylthiourea was also shown to have beneficial effects in ALF (Bruck et al. 1999; Guerrini 1994; Túnez et al. 2007).

Mild hypothermia improves the outcome of ALF in a wide range of experimental animal models (Cordoba et al.

1999; Rose et al. 2000; Schenker and Warren 1962; Traber et al. 1989; Jiang et al. 2009a; Bemeur et al. 2010b). While a number of mechanisms could explain this beneficial response, it is noteworthy that hypothermia has the potential to reduce free radical production (Globus et al. 1995). Jiang et al. (2009a) recently showed that mild hypothermia attenuates brain HO-1 mRNA and protein expression as well as eNOS and inducible NOS (iNOS) expression in the hepatic devascularized rat model of ALF. It was also shown that hypothermia led to normalization of eNOS mRNA expression in these animals (Sawara et al. 2009). Moreover, hypothermia delays the progression of encephalopathy, decreases proinflammatory cytokine levels, and increases GSH/GSSG ratios in the frontal cortex of mice with azoxymethane-induced ALF (Bemeur et al. 2010b).

To date, a limited number of studies have addressed the role of oxidative stress in the pathogenesis of HE in ALF patients. NAC was proven to be useful in the management of these patients (Harrison et al. 1991; Jones 1998; Wendon et al. 1994). Whether or not the beneficial effect of NAC relates simply to its antioxidant properties in the liver or to additional neuroprotective actions in the brain (NAC readily crosses the blood-brain barrier) remains to be established. Sodium benzoate, shown to be effective in the management of HE (Sushma et al. 1992) because of its ability to enhance the urinary excretion of ammonia (Brusilow et al. 1979), also has antioxidant properties (Haseloff et al. 1990; Upreti et al. 1991). Hypothermia has likewise been shown to be beneficial in humans with ALF due, at least in part, to its antioxidant effect (Jalan et al. 1999, 2003).

Chronic liver failure

NOS activity is increased in the brains of portacaval-shunted rats (Rao et al. 1995). iNOS and nNOS protein expression is increased in the brains of these animals (Rao 2002; Suárez et al. 2005; 2006) whereas brain eNOS protein expression is increased in TAA-induced cirrhosis in the rat (Hernández et al. 2004). An increase in HO-1 mRNA expression as well as a decrease in copper/zinc-SOD gene expression have been reported in the brains of portacaval-shunted rats (Song et al. 2002). Protein tyrosine nitration, a consequence of oxidative/nitrosative stress, was demonstrated in the cerebral cortex of these animals (Schliess et al. 2002; Suárez et al. 2006).

A distinctive neuropathologic finding in brains of patients with HE is the presence of Alzheimer type II astrocytes (Norenberg 1981). Characteristically, these cells contain increased amounts of lipofuscin pigment. These pigments consist of peroxidized lipids (Brunk 1989), consistent with their exposure to oxidative damage.

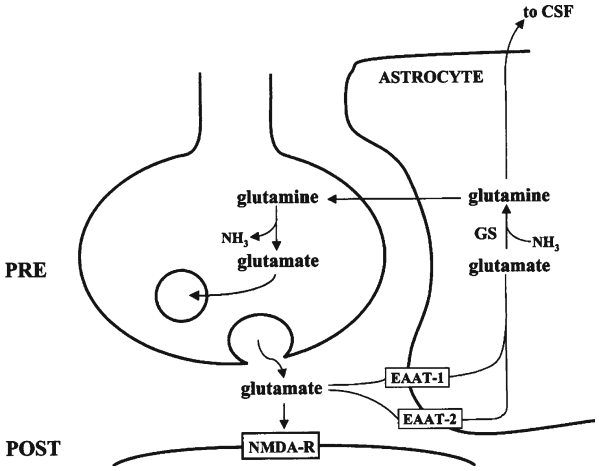

Fig. 2 Exposure of brain to increased ammonia results in down regulation of the astrocytic glutamate transporter EAAT-2 leading to increased extracellular glutamate concentrations which activates NMDA receptors (NMDAr). This activation could cause production of superoxide, nitric oxide, nitric oxide-dependent cGMP and protein tyrosine nitration which leads to oxidative/nitrosative stress. Tyrosine nitration of the major ammonia-removing enzyme glutamine synthetase (GS) then results in increased brain ammonia and a vicious cycle

Table 1 summarizes evidence for oxidative/nitrosative stress in the brain in relation to the pathogenesis of HE in liver failure.

Cellular localization of oxidative/nitrosative stress in hepatic encephalopathy

Evidence supports the view that astrocytes are a major source of reactive oxygen species under simulated HE conditions (Schliess et al. 2006). Glutamine synthetase is predominantly expressed in astrocytes and the glutamine synthetase inhibitor methionine sulfoximine (MSO) reduces protein tyrosine nitration in ammonia-treated rats (Master et al. 1999; Schliess et al. 2002). Immunohistochemical analysis of brain slices from ammonia-treated rats reveals considerable co-localization of 3′-nitrotyrosine with glutamine synthetase and glial fibrillary acidic protein, indicating protein tyrosine nitration in astrocytes (Schliess et al. 2002; Görg et al. 2003, 2007).

In a rat model of chronic liver failure (portacaval anastomosis), neurons were immunoreactive to nNOS whereas iNOS was expressed in pyramidal-like cortical neurons and perivascular astrocytes (Suárez et al. 2006). In the same animals, nitrotyrosine immunoreactivity was found in pyramidal-like cortical neurons and in perivascular astrocytes. It was also demonstrated that nNOS and iNOS are produced in the Purkinje (neuronal) cells and Bergmann glial cells in rats following portacaval anastomosis (Suárez et al. 2005). TAA-cirrhotic rats showed nNOS immunoreactivity in stellate and basket neurons and eNOS immuno-

Table 1 Oxidative/nitrosative stress in the brain in relation to the pathogenesis of hepatic encephalopathy in liver failure: review of the evidence

Animal model	Finding	Reference
Acute liver failure		
Hepatic devascularized rat	Increased HO-1 expression	Jiang et al. 2009a, b
	Increased eNOS expression	Jiang et al. 2009a, b
	Increased iNOS expression	Jiang et al. 2009a, b
	Increased nitrite/nitrate levels	Jiang et al. 2009a, b
	Increased eNOS mRNA expression	Sawara et al. 2009
Portacaval shunted rat with ammonia infusion	Increased brain nitric oxide production	Master et al. 1999
Thioacetamide-induced ALF in rat	Decreased catalase activity	Sathyasaikumar et al. 2007; Túnez et al. 2007
	Decreased glutathione peroxidase activity	Sathyasaikumar et al. 2007
	Increased rate of lipid peroxidation	Sathyasaikumar et al. 2007; Túnez et al. 2007
	Increased nitric oxide production	Sathyasaikumar et al. 2007
	Decreased GSH/GSSG	Sathyasaikumar et al. 2007
	Increased SOD activity	Sathyasaikumar et al. 2007
	Increased hydrogen peroxide production	Reddy et al. 2004
	Decreased total antioxidant status	Zarros et al. 2008
Azoxymethane-induced ALF in mouse	Increased HO-1 mRNA expression	Bemeur et al. 2009
	Decreased GSH/GSSG	Bemeur et al. 2010a, b
Chronic liver failure		
Portacaval shunted rat (4 weeks)	Increased HO-1 mRNA expression	Song et al. 2002
	Decreased Cu/Zn-SOD gene expression	Song et al. 2002
	Increased NOS activity	Rao et al. 1995
	Protein tyrosine nitration	Schliess et al. 2002
Portacaval shunted rat (1–6 months)	Increased iNOS protein expression	Suárez et al. 2005, 2006
	Increased nNOS protein expression	Suárez et al. 2005, 2006
	Protein tyrosine nitration	Suárez et al. 2006
Thioacetamide-induced cirrhosis in rat	Increased eNOS protein expression	Hernández et al. 2004

reactivity in perivascular glial cells of the white matter (Hernández et al. 2004). In the same experimental model of chronic liver failure, eNOS was located in Purkinje cell bodies and vessels endothelial cells (Hernández et al. 2004). Taken together, these data suggest that neuronal, glial and endothelial cells are all sources of nitric oxide in HE indicating that oxidative/nitrosative stress in HE is a multicellular phenomenon.

Oxidative/nitrosative stress in HE: ammonia or proinflammatory cytokines?

It has been proposed that oxidative/nitrosative stress results from NMDA-receptor activation (Fig. 2) (Hermenegildo et al. 1996; Kosenko et al. 1999; Marcaida et al. 1992). In a rodent model of ammonia neurotoxicity, Kosenko et al. (1997) showed decreased activity of several antioxidant enzymes, including glutathione peroxidase, manganese SOD and catalase. The same articles also described an increase in lipid peroxidation as well as glutathione depletion. The precise free radical specie(s) generated by ammonia is not certain, although increased production of superoxide (Kosenko et al. 1997, 2003) as well as increased nitric oxide production (Master et al. 1999) have been proposed. Hilgier et al. (2003) showed that infusion of ammonia into the striatum of rats resulted in the production of hydroxyl radicals. Moreover, ammonia toxicity increases the cerebral production of superoxide, nitric oxide and nitric oxide-dependent cGMP synthesis, as well as protein tyrosine nitration in an NMDA receptor-dependent manner (Hermenegildo et al. 1996; Kosenko et al. 1999, 2004; Master et al. 1999; Hermenegildo et al. 2000; Larsen et al. 2001; Schliess et al. 2002, 2006). However, evidence for similar mechanisms in the pathogenesis of HE in chronic liver failure *in vivo* is lacking.

In addition to ammonia, the inflammatory cascade generated by circulating and brain cytokines is also a

potential source of oxidative stress. It is well known that inflammation is associated with free radical production (Laroux et al. 2001; Bemeur et al. 2005). Bemeur et al. (2010a) showed that administration of the antioxidant NAC in an experimental model of ALF prevented the increase in cytokines and the concomitant decrease in brain GSH/GSSG in the frontal cortex. Protective effects of the anti-inflammatory drug minocycline were also reported in rats with ALF due to hepatic devascularization (Jiang et al. 2009b). It was proposed that synergism between the neurotoxic effects of ammonia and proinflammatory cytokines could explain the neurological complications in ALF Microglial activation and consequent oxidative/stress could be central to this synergism.

In summary, experimental evidence for oxidative/nitrosative stress in brain in experimental models of HE due to acute or chronic liver failure is increasing and several reports suggest that oxidative/nitrosative stress participates in the pathophysiologic cascade responsible for HE. Antioxidant therapy such as NAC is already being used in the management of ALF and its complications. Other antioxidants could prove to be a valuable adjunct to traditional HE therapies such as ammonia lowering strategies or anti-inflammatory drugs. Further studies are needed in order to assess these possibilities.

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