

## Review Article

# Neuro-oxidative-nitrosative stress in sepsis

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**Neuro-oxidative-nitrosative stress may prove the molecular basis underlying brain dysfunction in sepsis. In the current review, we describe how sepsis-induced reactive oxygen and nitrogen species (ROS/RNS) trigger lipid peroxidation chain reactions throughout the cerebrovasculature and surrounding brain parenchyma, due to failure of the local antioxidant systems. ROS/RNS cause structural membrane damage, induce inflammation, and scavenge nitric oxide (NO) to yield peroxynitrite (ONOO<sup>-</sup>). This activates the inducible NO synthase, which further compounds ONOO<sup>-</sup> formation. ROS/RNS cause mitochondrial dysfunction by inhibiting the mitochondrial electron transport chain and uncoupling oxidative phosphorylation, which ultimately leads to neuronal bioenergetic failure. Furthermore, in certain 'at risk' areas of the brain, free radicals may induce neuronal apoptosis. In the present review, we define a role for ROS/RNS-mediated neuronal bioenergetic failure and apoptosis as a primary mechanism underlying sepsis-associated encephalopathy and, in sepsis survivors, permanent cognitive deficits.**

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## Introduction

Sepsis, the systemic inflammatory response to infection (Bone, 1996), is a frequent cause of death, both in the general population and in patients in the intensive care unit (Vincent *et al*, 2006). In the latter setting, organ dysfunction, including acute brain

dysfunction, termed sepsis-associated encephalopathy (SAE) or sepsis-associated delirium, occurs in up to two thirds of patients (Vincent *et al*, 2006). SAE may be the first symptom of sepsis, and is characterized by confusion, agitation, and impaired consciousness (Young *et al*, 1990; Bolton *et al*, 1993; Ebersoldt *et al*, 2007; Iacobone *et al*, 2009). These symptoms may be an independent predictor of death (Sprung *et al*, 1990; Ebersoldt *et al*, 2007). Furthermore, encephalopathy in critically ill patients may predict long-term cognitive function after discharge from the intensive care unit (Girard *et al*, 2010). Accordingly, clinical and experimental studies suggest that up to 60% of sepsis survivors exhibit permanent cognitive deficits (Iwashyna *et al*, 2010), notably memory loss, and impaired learning capability (Hopkins *et al*, 1999, 2004, 2005; Barichello *et al*, 2005; Semmler *et al*, 2007).

As of now, the underlying mechanisms of SAE and the associated neurologic sequelae remain unresolved. However, as will be outlined in the present review, emerging evidence points toward a role for neuro-oxidative-nitrosative stress, that is, potentially damaging redox processes that involve the formation of reactive oxygen and nitrogen species (ROS/RNS)

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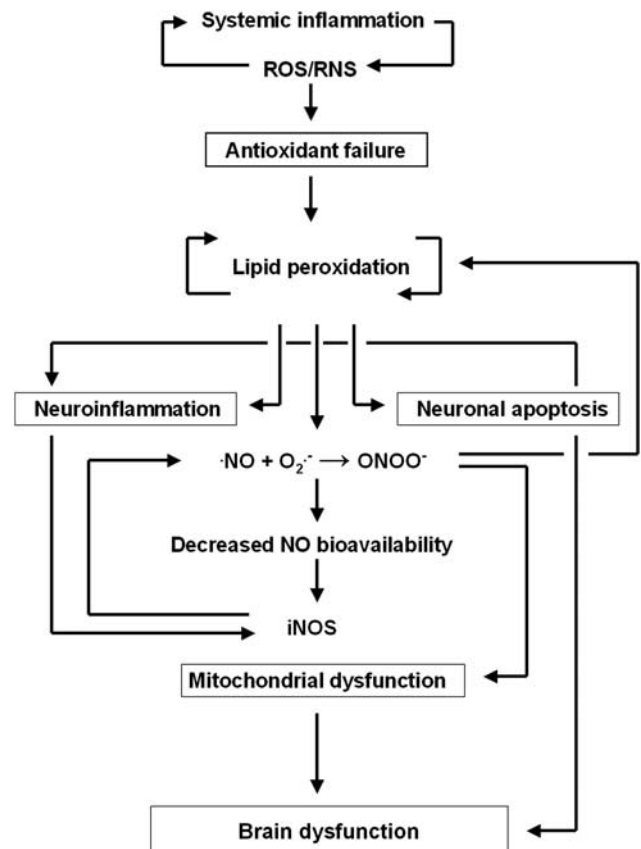
within the central nervous system. ROS include not only oxygen-centered free radicals, for example the superoxide anion ( $O_2^{\cdot-}$ ) and hydroxyl radicals ( $\cdot OH$ ), but also certain nonradical derivatives of oxygen, such as hydrogen peroxide ( $H_2O_2$ ), whereas nitric oxide (NO), peroxynitrite ( $ONOO^-$ ), and nitrogen dioxide ( $NO_2$ ) are examples of RNS (Halliwell, 2006). The brain is constantly exposed to relatively high levels of ROS and RNS, mainly due to a high metabolic rate of oxygen, which is associated with a high rate of  $O_2^{\cdot-}$  leakage from the mitochondrial electron transport chain (Halliwell, 2006). The abundance of eicosapentaenoic and docosahexaenoic polyunsaturated fatty-acid side chains in neuronal cell membranes, which are readily oxidized, force the limited antioxidant defenses to work at near-maximal levels, even in the healthy state (Halliwell, 2006).

Based on the data from various experimental models of sepsis in animals, as well as data from clinical studies, the present review will focus on the potential role of neuro-oxidative-nitrosative stress in the pathophysiology of sepsis. We propose a model, which comprises six principal components that follow in succession and are unified through the focal generation of ROS/RNS: (1) antioxidant failure, (2) lipid peroxidation, (3) inflammation, (4) NO depletion, (5) mitochondrial dysfunction, and (6) apoptosis. According to this model, ROS/RNS generated during the systemic inflammatory response initiate lipid peroxidation in the cerebrovasculature and brain parenchyma, due to failure of the antioxidant systems of the brain (Figure 1). Lipid peroxidation chain reactions generate further free radicals within the brain; the free radicals induce focal inflammation, decrease the vascular bioavailability of NO, and prompt neuronal bioenergetic failure through mechanisms established *vide infra*. This impairs cerebral oxidative metabolism and causes an acute suppression of global cerebral function, which then gives rise to the symptoms characteristic of SAE. Free radicals further induce neuronal apoptosis in certain brain regions 'at risk,' and thus cause cognitive deficits that may persist after clinical recovery from sepsis.

We hypothesize that this model provides the molecular basis for the brain dysfunction characteristic of sepsis. In the following, we review the current evidence that forms the basis of the six principal components of the model as proposed.

## Antioxidant failure

In the healthy state, free radical formation is counteracted by a variety of antioxidant systems that include the enzymes superoxide dismutase, catalase and glutathione peroxidase, as well as nonenzymatic, chain-breaking antioxidants, such as ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E) (Buettner, 1993; Halliwell, 2006).



**Figure 1** A hypothetical model of brain dysfunction in sepsis. Reactive oxygen and nitrogen species (ROS/RNS), generated during the systemic inflammatory response, trigger lipid peroxidation chain reactions, due to the limited antioxidant capacity of the brain. Free radical-mediated structural membrane damage induces neuroinflammation; furthermore, free radicals (such as superoxide anion,  $O_2^{\cdot-}$ ) deplete ambient nitric oxide ( $\cdot NO$ ) in the cerebrovascular bed to form peroxynitrite ( $ONOO^-$ ). The consequently reduced vascular bioavailability of NO acts in concert with the local inflammatory response to increase the expression of the inducible NO synthase (iNOS).  $ONOO^-$  irreversibly inhibits the mitochondrial electron transport chain, which increases the mitochondrial release of free radicals. Free radical-mediated uncoupling of oxidative phosphorylation compounds the mitochondrial dysfunction, and renders the neuron in a state of 'cytopathic hypoxia,' thus leading to neuronal bioenergetic failure. Furthermore, free radicals trigger apoptosis by altering the intracellular  $Ca^{2+}$  homeostasis in the brain areas 'at risk,' such as the cerebral cortex and hippocampus, which exacerbates the local inflammatory response further.

## Superoxide Dismutase, Catalase, and Glutathione Peroxidase

Superoxide dismutase activity has been reported to increase in the brains of experimentally septic rats (Barichello *et al*, 2006; Ninkovic *et al*, 2006). This enzyme dismutates  $O_2^{\cdot-}$  to  $H_2O_2$  and oxygen (Halliwell, 2006). In contrast to  $O_2^{\cdot-}$ ,  $H_2O_2$  diffuses readily across membranes into the extracellular compartment, in which it may decompose to  $\cdot OH$ . The latter has the highest known one-electron

**Table 1** Reactivity of selected reactive oxygen and nitrogen species (ROS/RNS) and antioxidants of relevance in neuro-oxidative-nitrosative stress

Couple		$E^{\circ}$ (mV)
Hydroxyl radical	[•OH, H <sup>+</sup> /H <sub>2</sub> O]	+2,310
Peroxynitrite/nitrogen dioxide	[ONOO <sup>-</sup> , 2 H <sup>+</sup> /NO <sub>2</sub> <sup>•</sup> , H <sub>2</sub> O]	+1,400
Nitrogen dioxide/nitrite	[NO <sub>2</sub> <sup>•</sup> /NO <sub>2</sub> <sup>-</sup> ]	+990
Lipid alkylperoxyl radical/lipid hydroxyperoxide	[L-OO•, H <sup>+</sup> /L-OOH]	+750–1,400
Superoxide anion/hydrogen peroxide	[O <sub>2</sub> <sup>•-</sup> , 2 H <sup>+</sup> /H <sub>2</sub> O <sub>2</sub> ]	+940
Lipid pentadienyl radical/bisallylic hydrogen group	[L•, H <sup>+</sup> /L-H]	+600
Nitric oxide/nitroxyl anion	[•NO/NO <sup>-</sup> ]	+390
Tocopheroxyl radical/ $\alpha$ -tocopherol	[ $\alpha$ T•, H <sup>+</sup> / $\alpha$ TH]	+500
Hydrogen peroxide/hydroxyl anion	[H <sub>2</sub> O <sub>2</sub> , H <sup>+</sup> /H <sub>2</sub> O, •OH]	+320
Ascorbate/ascorbyl radical	[AH/H•, A• <sup>-</sup> ]	+282

A higher one-electron reduction potential ( $E^{\circ}$ ) indicates higher reactivity; hence, a compound is thermodynamically capable of abstracting an electron from any biomolecule with a lower  $E^{\circ}$ . The reported  $E^{\circ}$  values have been obtained from Stanbury (1989); Koppenol *et al* (1992); Buettner (1993); and Bartberger *et al* (2002).

potential ( $E^{\circ}$ ), which places it at the top of the so-called 'league of reactivity' (Buettner, 1993) (Table 1), that is, it is thermodynamically capable of abstracting a hydrogen atom from any other biomolecule. Catalase and glutathione peroxidase prevent the formation of •OH by reducing H<sub>2</sub>O<sub>2</sub> to water and oxygen at the expense of glutathione (Halliwell, 2006). Thus, an increase in superoxide dismutase may be associated with an increase in oxidative stress, unless it is accompanied by a concurrent increase in catalase and glutathione peroxidase. Increased levels of brain superoxide dismutase without a proportional increase in catalase activity have thus been suggested to be a characteristic of brain regions that are susceptible to free radical-mediated structural membrane damage in sepsis (Barichello *et al*, 2006). Since catalase is not present in the brain mitochondria, where most of the H<sub>2</sub>O<sub>2</sub> is generated (Halliwell, 2006), the relative increase in mitochondrial glutathione peroxidase activity may prove a more accurate marker. Accordingly, melatonin, which increases the activity of mitochondrial glutathione peroxidase, has been demonstrated to reduce neuronal damage in experimentally septic rats (Sener *et al*, 2005b).

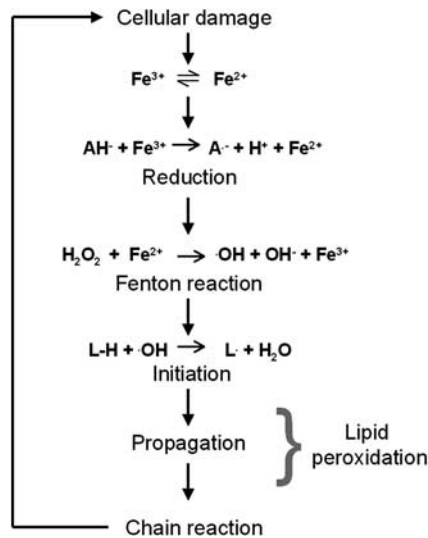
### Ascorbate and $\alpha$ -Tocopherol

The aqueous-phase antioxidant ascorbate prevents lipid peroxidation chain reactions by undergoing oxidation to yield the ascorbyl radical, a relatively inert intermediate that can donate an electron to any oxidizing species, given the low  $E^{\circ}$  of the ascorbate-ascorbyl radical couple (Table 1). Also, ascorbate recycles the tocopheroxyl radical back to the fully functional lipid-soluble  $\alpha$ -tocopherol isomer;  $\alpha$ -tocopherol acts as an antioxidant by chain-breaking lipid peroxides at the membrane-water interface in a manner similar to ascorbate (Sharma and Buettner, 1993).

In a study of patients with SAE, cerebrospinal fluid and plasma ascorbate levels were found to be

lower than in nonseptic controls, whereas  $\alpha$ -tocopherol levels were not affected (Voigt *et al*, 2002). This was interpreted to reflect ascorbate oxidation at rates that overwhelmed the capacity of the brain cells to regenerate ascorbate (Voigt *et al*, 2002). Since ascorbate was not found to be entirely depleted from the cerebrospinal fluid, it is, however, probably not the lack of antioxidant protection from ascorbate that facilitates neuro-oxidative-nitrosative stress in sepsis; neuro-oxidative-nitrosative stress is more likely to involve the often overlooked ability of ascorbate to catalyze, as opposed to quench, lipid-derived free radicals by reducing ferric (Fe<sup>3+</sup>) to ferrous (Fe<sup>2+</sup>) iron (Bailey, 2005) (Figure 2). Although the brain is rich in nonheme iron (Haacke *et al*, 2005), the potential for these reactions to occur is remote in healthy conditions, since iron is sequestered in noncatalytic protein-bound forms, such as ferritin (Haacke *et al*, 2005). In the context of sepsis, however, degenerating neurons and other cells could potentially release Fe<sup>2+</sup> to the extracellular space, and thus enable ascorbate to catalyze the formation of •OH through the Fenton reaction (Figure 2). A similar molecular concept has been demonstrated in patients undergoing lower limb vascular surgery with obligatory ischemia-reperfusion, due to cross-clamping, which increases the extracellular availability of Fe<sup>2+</sup> (Bailey *et al*, 2006). The patients were randomized to ascorbate prophylaxis or placebo in a double-blinded, randomized manner. During lower limb ischemia, the patients receiving ascorbate exhibited a *release* of lipid hydroperoxide across the ischemic vascular bed, and the systemic levels of lipid-derived alkoxy and alkyl radicals *increased* during reperfusion (Bailey *et al*, 2006). Analogous mechanisms may contribute to oxidative-nitrosative stress upon ischemia-reperfusion of the cerebrovascular bed, as indicated by findings from patients undergoing carotid endarterectomy (Bailey *et al*, 2007).

The concept of ascorbate as a prooxidant does, however, remain highly controversial, and there is evidence indicating that ascorbate retains its potent



**Figure 2** Ascorbate: the phantom menace? Catalytically active ferric ( $\text{Fe}^{3+}$ ) and ferrous ( $\text{Fe}^{2+}$ ) iron may be unlocked and released to the extracellular space upon cellular damage. Because of its relatively low one-electron reduction potential ( $E^{\circ} = +282 \text{ mV}$ ), ascorbate ( $\text{AH}^-$ ) is capable of donating an electron to  $\text{Fe}^{3+}$  thereby reducing it to  $\text{Fe}^{2+}$ , which in turn reacts with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to form the hydroxyl radical ( $\bullet\text{OH}$ ) through the Fenton reaction.  $\bullet\text{OH}$  may consequently initiate lipid peroxidation chain reactions and cause structural membrane damage throughout the cerebrovasculature and brain parenchyma.  $\text{A}\bullet^-$ , ascorbyl radical;  $\text{L}\bullet$ , lipid pentadienyl radical;  $\text{OH}^-$ , hydroxide.

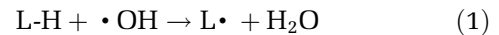
antioxidant properties in plasma *in vivo*, even in the presence of iron overload (Chen *et al*, 2000). Therefore, the relevance of these iron-dependent mechanisms in the brain in sepsis remains to be elucidated, but is supported by findings from septic rats, in which treatment with the glutathione precursor *N*-acetylcysteine only improved long-term cognitive outcome when combined with the iron-chelating agent desferrioxamine, although neither antioxidant had a significant effect when administered alone (Barichello *et al*, 2007).

We propose that failure of the antioxidant defenses in the brain is an early event in sepsis that facilitates oxidative-nitrosative stress.

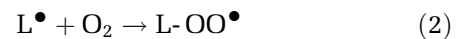
## Lipid peroxidation

The inadequacy of the antioxidant response renders the brain susceptible to oxidation in sepsis. Oxidative stress is initially promoted by phagocytic cells, such as neutrophils, which generate disproportionately large amounts of ROS/RNS upon an infectious insult; this mechanism probably serves to eradicate invading pathogens (Martins *et al*, 2003). Furthermore, the invading pathogen may release endo- and exotoxins, including prooxidants such as  $\text{H}_2\text{O}_2$  (Berpohl *et al*, 2005), which cause cellular damage

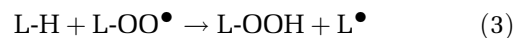
and facilitate the formation of ROS/RNS. ROS/RNS are thermodynamically poised to cause cell membrane damage throughout the cerebrovasculature and brain parenchyma (Boueiz and Hassoun, 2009; Pun *et al*, 2009), due to the relatively low  $E^{\circ}$  of the bisallylic hydrogen group (L-H) in polyunsaturated fatty-acid side chains (Table 1). Any oxidizing species with a higher  $E^{\circ}$  than L-H can thus initiate lipid peroxidation;  $\bullet\text{OH}$  is used as an initiator in this example:



The resultant pentadienyl radical ( $\text{L}\bullet$ ) reacts rapidly with oxygen ( $k = 3 \times 10^8/\text{M s}$ ) to form an alkylperoxyl radical ( $\text{L-OO}\bullet$ ):



$\text{L-OO}\bullet$  has a higher  $E^{\circ}$  than does L-H (Table 1), thus enabling it to propagate the reaction to yield lipid hydroperoxide ( $\text{L-OOH}$ ) and additional  $\text{L}\bullet$ :



The  $\text{L}\bullet$  may then generate more  $\text{L-OO}\bullet$ ; in effect, a lipid peroxidation chain reaction ensues, in which surrounding membrane lipids are oxidized. This increases membrane fluidity and triggers inactivation of membrane-bound proteins, thus impairing membrane integrity and function (Halliwell, 2006). Through these mechanisms, free radicals may contribute to the widespread blood-brain barrier damage that has been demonstrated in experimental studies of sepsis (Eckman *et al*, 1958; Clawson *et al*, 1966; du Moulin *et al*, 1985; Esen *et al*, 2005). Accordingly, treatment with  $\beta$ -glucan has been found to reduce vasogenic edema and hemorrhage in the brains of experimentally septic rats (Sener *et al*, 2005a), presumably by preventing blood-brain barrier disruption.  $\beta$ -glucan is an antioxidant derived from cell walls of fungi and some cereal plants; it is unknown how it exerts its antioxidant effects, but it is thought to bind to the plasma membrane lipid bilayer and specifically inhibit lipid peroxidation in a manner similar to that of  $\alpha$ -tocopherol (Babincova *et al*, 2002).

Until now, no studies have directly demonstrated lipid peroxidation in sepsis, either in the cerebrovascular endothelium, or in the brain parenchyma. There is, however, some indirect evidence of its presence, since lipid peroxides degrade to reactive aldehyde products, such as malondialdehyde. Malondialdehyde belongs to the group of thiobarbituric acid reactive substances (TBARS), meaning that it is capable of reacting with thiobarbituric acid to yield a fluorescent product than can be detected *ex vivo* (Moore and Roberts, 1998). The concentration of TBARS increases in cerebrospinal fluid of patients with sepsis (Takezawa *et al*, 1983) and in the brain homogenates of septic animals (Takeda *et al*, 1986; Abd El-Gawad and Khalifa, 2001; Sener *et al*, 2005a, b; Goraca and Aslanowicz-Antkowiak, 2009). Elevated TBARS levels have furthermore been

demonstrated in specific brain regions, that is, the hippocampus, the cerebral cortex, and the cerebellum, as early as 6 hours after the induction of experimental sepsis in rats (Barichello *et al*, 2006).

It should be noted that the available evidence on sepsis-associated lipid peroxidation in the brain is almost exclusively based on TBARS measurements. Although the measurement of TBARS works well when applied to well-defined membrane systems *in vitro*, its application to body fluids and tissue extracts is problematic (Moore and Roberts, 1998). TBARS assays are relatively nonspecific, since aldehydes other than malondialdehyde are present in body fluids, many of which form chromogens that may yield false-positive readings. Furthermore, the TBARS assays may in themselves produce TBARS, due to the decomposition of lipid peroxides. Consequently, these indirect findings must be interpreted with caution. Electron paramagnetic resonance spectroscopy is the only technique that exists for the direct molecular detection and subsequent characterization of free radicals *in vivo* (Holley and Cheeseman, 1993). This technique has not yet been applied to investigate neuro-oxidative-nitrosative stress in sepsis.

We propose that free radical-mediated lipid peroxidation causes structural membrane damage in the cerebrovasculature and brain parenchyma from the very early stages of sepsis.

## Inflammation

In sepsis, the widespread structural membrane damage throughout the cerebrovascular endothelium and surrounding brain parenchyma is accompanied by a local inflammatory response with increased intrathecal levels of cytokines (Sacoccio *et al*, 1998). Tumor necrosis factor (TNF)- $\alpha$  expression increases, initially in the circumventricular organs and subsequently in glial cells throughout the brain parenchyma (Gatti and Bartfai, 1993; Pitossi *et al*, 1997; Nadeau and Rivest, 2000; Semmler *et al*, 2008); this may facilitate the glial expression of several other cytokines 'downstream' of TNF- $\alpha$ , such as interleukin (IL)-1 $\beta$  and IL-6 (Pitossi *et al*, 1997; Meyer *et al*, 1997; Satta *et al*, 1998; Semmler *et al*, 2008).

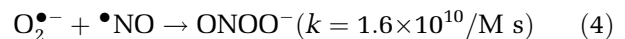
No studies have yet succeeded in disassociating oxidative-nitrosative stress from inflammation in the brain during systemic inflammation. Nevertheless, the biological half-lives of ROS/RNS are relatively short when compared with those of cytokines; the half-lives of ROS/RNS are as low as  $10^{-9}$  seconds for  $\bullet$ OH (Sies, 1993), whereas the reported half-lives of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, range from 6 to 20 minutes (Tracey and Cerami, 1993). This may favor free radicals as *inducers*, rather than mere *propagators* of inflammation. Furthermore, free radical-mediated blood-brain barrier disruption may allow noxious metabolites to enter the brain parenchyma and thus compound inflammation.

We propose that the release of free radicals and free radical-mediated structural membrane damage is an upstream signal that induces focal inflammation in the brain in sepsis.

## NO depletion

In the brain, the gaseous signaling molecule NO serves a number of cellular functions, including neurotransmission, regulation of blood-vessel tone, and immunity (Pacher *et al*, 2007). NO, an endogenous vasodilator, is produced from arginine by the members of the NO synthase (NOS) family of proteins, that is neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (Pacher *et al*, 2007). Whereas nNOS and eNOS are constitutively expressed and require the formation of  $\text{Ca}^{2+}$ -calmodulin complexes for their activation, iNOS exerts its activity in a  $\text{Ca}^{2+}$ -independent manner.

Any free radical, for instance  $\text{O}_2^{\bullet-}$ , lipid-derived alkoxyl free radical (LO $\bullet$ ) or  $\bullet$ OH may react with NO to yield ONOO $^-$  in a diffusion-controlled manner (Nausner and Koppenol, 2002):



Hence, free radicals may deplete NO through the formation of ONOO $^-$ , thus decreasing the vascular bioavailability of NO. Data from clinical studies support that the bioavailability of NO is decreased in sepsis (Doerschug *et al*, 2007; Davis *et al*, 2009), which, combined with endothelial damage, may contribute to the inhomogeneity of microvascular perfusion that was recently demonstrated in septic sheep (Taccone *et al*, 2010). This may furthermore impair neurovascular coupling (Rosengarten *et al*, 2009) and dynamic cerebral blood flow autoregulation (Pfister *et al*, 2008; Steiner *et al*, 2009), and thus prompt focal ischemia/hypoxia and blood-brain barrier damage upon acute surges in blood pressure. Changes in the levels of various vasoactive peptides that are involved in the regulation of cerebrovascular resistance (Pittet *et al*, 1991; Linscheid *et al*, 2004; Berg *et al*, 2009), as well as a reduced deformability of red blood cells due to oxidative modifications, which complicates their passage through the cerebral microvessels (Papadopoulos *et al*, 2000), may contribute to focal cerebral hypoxia. Since hypoxia compounds mitochondrial free radical formation (Guzy and Schumacker, 2006), this forms the basis for a vicious cycle, in which the free radical-mediated depletion of NO is maintained. Accordingly, we recently reported evidence of a similar vicious cycle during acute inspiratory hypoxia in healthy humans (Bailey *et al*, 2009).

In the healthy state, iNOS activation is low, but this enzyme may be induced in glial cells and increase NO production in response to a variety of noxious stimuli (Pacher *et al*, 2007), such as TNF- $\alpha$  and IL-1 $\beta$  (Wong *et al*, 1996; Alexander *et al*, 2008)

and, possibly, decreased ambient NO levels (Galijašević *et al*, 2003). A decreased NO bioavailability may therefore act in concert with focal inflammation to enhance iNOS expression. Thus, in experimentally septic rats, iNOS messenger RNA levels increase in vascular, glial, and neuronal structures throughout the brain (Wong *et al*, 1996; Jacobs *et al*, 1997; Harada *et al*, 1999), and levels of the NO metabolites, nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ), both of which reduce to evolve NO, have been found to be elevated in the brain parenchyma and cerebrospinal fluid (Wong *et al*, 1996; Abd El-Gawad and Khalifa, 2001). Moreover, one study has provided *in vivo* evidence for increased intrathecal NO formation in septic rats using electron paramagnetic resonance combined with an NO spin-trapping technique with an iron and *N,N*-diethyldithiocarbamate complex (Suzuki *et al*, 1998).

Although it may seem counterintuitive, an enhanced iNOS-dependent NO production may coincide with a decreased bioavailability of NO, since NO is rapidly depleted when free radicals are present (equation (4); Table 1). Therefore, iNOS is likely to enhance oxidative-nitrosative stress through the formation of ONOO<sup>-</sup>. ONOO<sup>-</sup> is much more reactive than NO (Table 1), causes tyrosine nitration and cysteine oxidation in various proteins (Radi *et al*, 1991), and decomposes to highly toxic free radicals, such as  $\text{NO}_2^\bullet$  and  $\bullet\text{OH}$  (Buettner, 1993; Bartberger *et al*, 2002). Cerebrospinal fluid levels of 3-nitrotyrosine have recently been found to be elevated in children with sepsis (Hamed *et al*, 2009); 3-nitrotyrosine, which is formed when a tyrosyl radical combines with  $\text{NO}_2^\bullet$  during tyrosine nitration (Pacher *et al*, 2007), is relatively stable, and can thus be considered a surrogate molecular 'footprint' of nitrosative stress.

We propose that free radical-mediated NO depletion decreases the cerebrovascular bioavailability of NO in sepsis, a reaction which is further fuelled by focal hypoxia and the upregulation of iNOS; the resultant generation of ONOO<sup>-</sup> augments oxidative-nitrosative stress by decomposing to other highly reactive free radicals.

## Mitochondrial dysfunction

The presence of an enhanced NO- and free radical-driven ONOO<sup>-</sup> production within the brain may prove critical to neuronal function in sepsis. NO donors, which yield NO and subsequently ONOO<sup>-</sup>, induce a rapid decrease in oxygen consumption by inhibiting complex I and IV of the mitochondrial electron transport chain when added to cell cultures and intact mitochondria (Clementi *et al*, 1998; Brookes *et al*, 1999). NO reversibly inhibits mitochondrial respiration by competing with oxygen for the binding site at complex I, whereas ONOO<sup>-</sup> attenuates electron transport both at complex I (Cassina and Radi, 1996; Clementi *et al*, 1998), and

to a lesser extent at complex IV (Brookes *et al*, 1999) through irreversible oxidative modifications. This may explain the presence of impaired complex I and complex IV activities which have been demonstrated in experimentally septic rats (Chuang *et al*, 2002; Comim *et al*, 2008; d'Avila *et al*, 2008).

## $\text{O}_2^{\bullet-}$ Formation

Impaired complex I and complex IV activities should theoretically cause a marked increase in the leakage of  $\text{O}_2^{\bullet-}$  from the electron transport chain (Riobo *et al*, 2001). Direct evidence demonstrating an increased mitochondrial  $\text{O}_2^{\bullet-}$  production is, however, limited. It has been reported that the  $\text{O}_2^{\bullet-}$  formation increases in the brain stem tissue of experimentally septic rats, as determined by luminometry after incubation of the tissue in a lucigenin-containing HEPES buffer (Chuang *et al*, 2003). Likewise, the  $\text{O}_2^{\bullet-}$  content, measured by the reduction of nitroblau-tetrazolium, has been found to increase in the brain capillaries of experimentally septic rats (Ninkovic *et al*, 2006). However, no extracellular  $\text{O}_2^{\bullet-}$  could be detected in the brains of experimentally septic pigs by use of the same method for  $\text{O}_2^{\bullet-}$  measurements (Deutschman *et al*, 1990), most likely because the negative charge of  $\text{O}_2^{\bullet-}$  principally restricts it to the intracellular compartment.

## Mitochondrial Uncoupling

Efficient oxidative metabolism depends on the coupling of electron transport and oxidative phosphorylation through a proton gradient across the inner mitochondrial membrane.  $\text{O}_2^{\bullet-}$  and various lipid peroxides are able to activate uncoupling proteins, which increase proton permeability across the inner mitochondrial membrane (Crompton, 1999; Echtay *et al*, 2002). This allows protons to pass into the mitochondrial matrix to join electrons in the form of oxide ions ( $\text{O}_2^{\bullet-}$ ) to form water in an exothermic reaction and without the generation of ATP from ADP. Uncoupling of oxidative phosphorylation was recently demonstrated in the brain mitochondria of septic mice (d'Avila *et al*, 2008). Although mitochondrial uncoupling may diminish the generation of ROS by neutralizing electrons that leak from the electron transport chain, it may also cause *cytopathic hypoxia*, which signifies that mitochondria are unable to use oxygen, regardless of whether it is available or not (Fink, 2001). Additionally, ONOO<sup>-</sup> may introduce single-strand breaks in DNA, thus triggering the activation of various repair processes, including the nuclear NAD<sup>+</sup>-consuming enzyme poly(ADP-ribose) polymerase, which increases the cellular metabolic requirements (Zhang *et al*, 1994). Combined with a state of cytopathic hypoxia, this may prompt neuronal bioenergetic failure. Accordingly, the global cerebral metabolic rate of oxygen has been found to be reduced in patients with severe

sepsis (Maekawa *et al*, 1991). However, sedation remains a potential source of error in such clinical studies. In contrast, the cerebral metabolic rate of oxygen has been found to be largely unaffected in healthy humans subjected to experimental sepsis by means of lipopolysaccharide infusion (Møller *et al*, 2002; Berg *et al*, 2010). Since, however, these healthy volunteers did not exhibit any changes in the level of consciousness, the relevance of such observations for critically ill septic patients showing signs of encephalopathy is unclear. As of now, regional cerebral oxidative metabolism has not been assessed in humans, either in experimental or clinical sepsis.

We propose that oxidative-nitrosative stress causes a global cerebral dysfunction in sepsis by prompting neuronal bioenergetic failure, and, consequently, impairing cerebral oxidative metabolism.

## Apoptosis

Apart from bioenergetic failure, free radicals may induce mitochondria-dependent apoptosis, by impairing membrane integrity and function in mitochondria and the endoplasmic reticulum (Polster and Fiskum, 2004). This increases intracellular  $\text{Ca}^{2+}$  (Satrustegui and Richter, 1984), which induces permeability transition pores in neuronal mitochondria (Crompton, 1999). Cytochrome *c*, which transports electrons from complex III to IV, is consequently translocated from the inner mitochondrial membrane to the cytoplasm (Brustovetsky *et al*, 2002). This translocation is considered a key step in the initiation of mitochondria-dependent apoptosis, since cytochrome *c*, once present in the cytoplasm, comprises an upstream signal in the apoptotic cascade (Polster and Fiskum, 2004). The presence of mitochondria-dependent neuronal apoptosis in sepsis is supported by studies showing increased free intracellular  $\text{Ca}^{2+}$  (Zhan *et al*, 1996) and enhanced cytoplasmic cytochrome *c* immunoreactivity (Messaris *et al*, 2004) in experimentally septic animals. Accordingly, apoptotic neurons with degenerating mitochondria have been observed in several brain regions of experimentally septic animals and deceased septic patients, including the cerebral cortex and hippocampus (Papadopoulos *et al*, 1999; Vereker *et al*, 2000; Semmler *et al*, 2005; Barichello *et al*, 2006; Orihuela *et al*, 2006; Kafa *et al*, 2010), the cerebellum (Messaris *et al*, 2004; Barichello *et al*, 2006), and some autonomic centers in the brain stem (Sharshar *et al*, 2003, 2004).

## Intrinsic and Extrinsic Factors

The underlying mechanisms that render some brain regions more susceptible to apoptosis than others are largely unknown, but likely involve both intrinsic and extrinsic factors. For example, the hippocampus has been found to be particularly vulnerable to

apoptosis when compared with other brain regions in experimentally septic rats (Semmler *et al*, 2005); even so, the regional cerebral metabolic rate of oxygen in the hippocampus is not remarkably high (Yamaguchi *et al*, 1986), nor does it contain large amounts of iron (Haacke *et al*, 2005). Rather, other intrinsic properties, such as its relatively high content of docosahexaenoic acid (McNamara and Carlson, 2006), as well as an insufficient antioxidant capacity (Barichello *et al*, 2006), are likely to render this region 'at risk' of free radical-induced apoptosis.

Extrinsic factors, such as cerebral ischemia and/or hemorrhage may prime a given region to apoptosis and render it 'at risk' by compounding free radical-mediated damage. Based on a multiwavelength near-infrared spectroscopy technique, a hypoxia-dependent reduction of the mitochondrial complex IV has been reported in experimentally septic rats (Schaefer and Biber, 1993). Furthermore, multiple ischemic zones and hemorrhages are frequent findings in autopsy studies of patients dying from septic shock (Sharshar *et al*, 2003, 2004). These findings may for instance be explained by microvascular failure with an impaired cerebral blood flow autoregulation, thus causing alternating regional hypo- and hyperperfusion in response to fluctuations in the cerebral perfusion pressure. Another mechanism that may be involved is ROS/RNS-mediated contraction of capillary pericytes, which has previously been found to compromise microvascular perfusion in experimental stroke (Yemisci *et al*, 2009).

Whereas a cerebral hemorrhage may prime a brain region to oxidative-nitrosative stress by increasing the extracellular availability of  $\text{Fe}^{2+}$ , ischemia may increase the leakage of electrons from the mitochondrial complex III (Guzy and Schumacker, 2006), thus leading to increased free radical formation. This 'hypoxia priming' could explain the presence of apparently randomly scattered groups of degenerating neurons in the cortex of experimentally septic pigs (Papadopoulos *et al*, 1999). However, the relationship between histological signs of ischemia and apoptosis are not clearcut in the autopsy studies of deceased septic patients, and markers of oxidative-nitrosative damage have not been assessed (Sharshar *et al*, 2003, 2004).

Neuronal apoptosis was found to be associated with an enhanced endothelial iNOS expression in deceased septic patients (Sharshar *et al*, 2003, 2004), and in experimentally septic rats, this was found to be particularly pronounced in the hippocampus (Semmler *et al*, 2005). Furthermore, neuronal apoptosis in this area was markedly reduced upon iNOS inhibition (Semmler *et al*, 2005). This implies that the degree of iNOS upregulation may reflect the severity of oxidative-nitrosative stress upon various intrinsic and extrinsic factors in a given brain region, and further supports the notion that iNOS is involved as a critical propagator of oxidative-nitrosative stress.

## Cognitive Decline

Longitudinal studies of cognitive function in sepsis survivors and experimental studies in rodents may shed some light on the clinical impact of neuronal apoptosis. Elderly sepsis survivors were recently reported to exhibit a pronounced cognitive decline when compared with nonseptic patients at 8-year follow-up (Iwashyna *et al*, 2010). In another study, a global intellectual decline was found to be present in patients who had recovered from severe sepsis complicated by adult respiratory distress syndrome (Hopkins *et al*, 1999). In this cohort, most of the cognitive deficits were spontaneously reversed 1 year after discharge (Hopkins *et al*, 1999); however, some impairment of the hippocampus-dependent processes tended to persist in the majority of patients (Hopkins *et al*, 1999, 2004) and did not cease at 2-year follow-up (Hopkins *et al*, 2005).

Data from animal studies also indicate that hippocampus-dependent learning and memory is permanently attenuated after a septic episode (Barichello *et al*, 2005, 2007; Semmler *et al*, 2007; Weberpals *et al*, 2009). This is likely to involve apoptosis of neuronal progenitor cells within the dentate gyrus. These cells are critical to memory processing and formation (Aimone *et al*, 2006) and rapidly undergo apoptosis upon H<sub>2</sub>O<sub>2</sub>-induced oxidative stress *in vitro* (Braun *et al*, 2002).

In rats surviving sepsis, permanent cognitive deficits were alleviated by the combined antioxidant treatment with *N*-acetylcysteine and desferrioxamine (Barichello *et al*, 2007). Thus, learning and memory performance, as evaluated by inhibitory avoidance and open field habituation tests, was impaired to a lesser extent in treated animals after recovery from sepsis (Barichello *et al*, 2007). Moreover, *N*-acetylcysteine and desferrioxamine decreased the hippocampal content of protein carbonyls, indicators of protein oxidation (Barichello *et al*, 2007). Likewise, iNOS gene deficiency appears to protect experimentally septic mice from permanent cognitive deficits (Weberpals *et al*, 2009), again supporting the role of iNOS as a critical propagator of oxidative-nitrosative stress. To validate the concept of free radical generation being responsible for regional neuronal apoptosis and impaired cognitive function, further studies are required of the time course, magnitude, and reversibility of either process alone, as well as their interrelation, in particular in patients with (rather than in experimental models of) sepsis.

We propose that free radicals cause apoptosis in selected brain regions and that this contributes to the permanent cognitive deficits that are commonly encountered in sepsis survivors.

## Complementary mechanisms

Brain dysfunction in sepsis may involve a number of alternative mechanisms, which have been reviewed

extensively elsewhere (Papadopoulos *et al*, 2000; Ebersoldt *et al*, 2007; Iacobone *et al*, 2009), and will only briefly be outlined here. These mechanisms and the six principal components of the oxidative-nitrosative stress cascade proposed above are not mutually exclusive, and may to different extents interact to affect brain function in sepsis.

Components of the innate immune system, such as proinflammatory cytokines and complement may affect blood–brain barrier function directly. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 attenuate blood–brain barrier integrity *in vitro*, assessed by electrical resistance and electron microscopy of rat cerebrovascular endothelial cells (Duchini *et al*, 1996; de Vries *et al*, 1996), and septic mice devoid of the TNF receptor 1 develop less cerebral edema compared with control mice (Alexander *et al*, 2008). By use of electron microscopy, it was similarly found that administration of an antibody to TNF- $\alpha$  protected blood-brain barrier integrity (Tsao *et al*, 2001). Likewise, treatment of septic rats with a neutralizing antibody to the complement anaphylatoxin C5a reduced breaching of the blood-brain barrier (Flierl *et al*, 2009).

Alterations in neurotransmitter signaling may cause brain dysfunction. Accordingly, various experimental studies suggest that sepsis is associated with abnormal cholinergic,  $\beta$ -adrenergic,  $\gamma$ -aminobutyric, serotonergic, and glutamatergic signaling (Winder *et al*, 1988; Shimizu *et al*, 1999; Kadoi and Saito, 1996; Kadoi *et al*, 1996; Semmler *et al*, 2007; Toklu *et al*, 2009). Furthermore, the combination of an increased peripheral protein breakdown, and the concomitant increased hepatic synthesis of acute-phase reactants may affect central noradrenergic signaling. From the very early stages of sepsis, this causes a decrease in the plasma ratio between branched-chain and aromatic amino acids, which compete for transport through the same saturable carrier across the blood-brain barrier (Basler *et al*, 2002; Berg *et al*, 2010). This may increase the intracerebral levels of aromatic amino acids, thereby facilitating the generation of ‘false’ neurotransmitters, such as  $\beta$ -phenylethylamine and octopamine, both of which inhibit noradrenergic pathways (Freund *et al*, 1978, 1979).

## Conclusion

Oxidative-nitrosative stress may contribute to brain dysfunction in sepsis, both in terms of SAE and permanent cognitive deficits. As of now, direct evidence for this hypothesis is, however, lacking in humans. Although brain dysfunction is frequently the initial symptom of sepsis, it is not an isolated phenomenon. As the clinical condition deteriorates and multiorgan failure evolves, brain function may decline further. Prompt and specific antibiotic therapy to eradicate the causative pathogen as well as supportive therapy to maintain and restore organ function in the septic patient are the most critical



measures to improve mortality and morbidity. However, there is currently no specific therapy for the treatment of brain dysfunction in sepsis. Therefore, the presence of neuro-oxidative-nitrosative stress is an attractive concept warranting further consideration. Future studies should include interventional experimental and clinical trials incorporating targeted delivery of nootropic 'radical scavengers' to the blood-brain barrier and brain parenchyma. Researchers would need to consider the physiochemical suitability of proposed neurotherapeutic agents since effective delivery is a complex outcome of the agent's lipophilicity, ionic charge, molecular mass, relative brain tissue/plasma binding affinities, and ability to activate endogenous transporters (Lo *et al*, 2001). Recent pharmacogenomic advances have led to the rapid development of novel strategies that promote effective delivery to the injured or susceptible brain (Scherrmann, 2002; Barber *et al*, 2009). Future incorporation of these 'hit compounds' to the central nervous system will clearly facilitate a more comprehensive examination of the role that free radicals and associated reactants have in the pathophysiology of brain dysfunction in sepsis.

## Author contributions

RMGB drafted the manuscript. KM and DMB made critical revisions. All authors read and approved the final manuscript.

## Disclosure/conflict of interest

The authors declare no conflict of interest.

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