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Review

The role of the nitric oxide pathway in brain injury and its treatment — From bench to bedside☆

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ABSTRACT

Nitric oxide (NO) is a key signalling molecule in the regulation of cerebral blood flow. This review summarises current evidence regarding the role of NO in the regulation of cerebral blood flow at rest, under physiological conditions, and after brain injury, focusing on subarachnoid haemorrhage, traumatic brain injury, and ischaemic stroke and following cardiac arrest. We also review the role of NO in the response to hypoxic insult in the developing brain. NO depletion in ischaemic brain tissue plays a pivotal role in the development of subsequent morbidity and mortality through microcirculatory disturbance and disordered blood flow regulation. NO derived from endothelial nitric oxide synthase (eNOS) appears to have neuroprotective properties. However NO derived from inducible nitric oxide synthase (iNOS) may have neurotoxic effects. Cerebral NO donor agents, for example sodium nitrite, appear to replicate the effects of eNOS derived NO, and therefore have neuroprotective properties. This is true in both the adult and immature brain. We conclude that these agents should be further investigated as targeted pharmacotherapy to protect against secondary brain injury.

Contents

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Introduction

Nitric oxide (NO) is a gaseous molecule synthesised from L-arginine by the enzyme nitric oxide synthase (NOS) (Stuehr and Griffith, 1992). It acts as a neurotransmitter and is a component of the signalling pathways that operate between cerebral blood vessels, neurons and glial cells. Three isoforms of NOS exist: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS). Disruption to the NO pathway underlies many of the mechanisms behind brain injury and NO produced from each of the three isoforms influences the evolution of brain damage in different ways (Toda et al., 2009). This article will explore the current evidence surrounding the role of the NO signalling pathway and NOS isoforms in the regulation of resting cerebral blood flow and in the neuroprotective and pathophysiological processes that occur after injury. We will also examine the potential for manipulation of this pathway as potential treatment strategy.

Nitric oxide synthase — expression and functions

Nitric oxide is derived from three isoforms of the enzyme NOS. The different sites of expression and activity of these enzymes play a critical role in the differing functions of NO.

eNOS is expressed in the vascular endothelium and choroid plexus. NO derived from eNOS (eNOS-NO) plays a role in preserving and maintaining the brain's microcirculation (Toda et al., 2009), inhibiting platelet aggregation, leukocyte adhesion and migration (Broos et al., 2011), and reducing smooth muscle proliferation (Toda et al., 2009).

nNOS is expressed in neuronal cell bodies and NO derived from nNOS (nNOS-NO) acts as an important neurotransmitter associated with neuronal plasticity, memory formation, regulation of central nervous system blood flow, transmission of pain signals and neurotransmitter release (Schuman and Madison, 1994; Toda and Okamura, 2003).

iNOS is expressed in macrophages, glial cells and tumour cells in response to pro-inflammatory cytokines or endotoxin (Marletta, 1988) and unlike eNOS and nNOS it is not expressed unless induced by cytokines or other agents. It can produce a large amount (100–1000 times greater) of NO in relation to eNOS and nNOS (Pautz et al., 2010), due to its independence from calcium dependent mechanisms for activation. After induction, iNOS continuously produces NO until the enzyme is degraded (MacMicking et al., 1997).

Role of NO in regulation of cerebral blood flow

The brain accounts for 20% of the body's energy consumption despite only accounting for 2% of its mass. One of the major roles of the cerebral circulation is to supply oxygen to the brain tissues as neuronal activation requires large amounts of energy to regulate the ion fluxes that occur on depolarisation. Therefore, it is essential that cerebral blood flow (CBF) is tightly regulated.

The NO signalling pathway plays a major role in the regulation of CBF at rest and during physiological and pathological stresses. Understanding the mechanisms behind the regulation of these processes may therefore give useful insights into the CBF changes that occur during cerebral injury.

Two main mechanisms underlie the regulation of CBF at rest, autoregulation and neurovascular coupling. eNOS-NO plays a key role in autoregulation, whereas nNOS derived NO appears crucial for neurovascular coupling.

Autoregulation

Autoregulation is the mechanism by which a consistent supply of blood to the brain tissues in the face of changing cerebral perfusion pressure (CPP) is maintained. eNOS derived NO is essential for extending the lower limit of autoregulation. This is demonstrated by a right shift of the autoregulation curve in eNOS knockout mice at low perfusion pressures (Huang et al., 1996) (see Fig. 1). Elevation of CBF near the lower limit of autoregulation and an increase in the hypotensive portion of the autoregulatory curve are also NO dependent (Jones et al., 2003). During pathological processes where CBF is reduced, such as subarachnoid haemorrhage or ischaemic stroke, NO depletion could therefore further exacerbate decreased flow conditions and increase the likelihood of permanent neuronal damage.

Neurovascular coupling

Neurovascular coupling is the process by which increased neuronal activity is linked to the local regulation of CBF. This ensures that the supply of oxygen is always greater than the demand, and is also known as functional hyperaemia. It is achieved via an integrated action of neurons, glial cells and blood vessels that form a 'neurovascular unit' (Attwell et al., 2010). Several signalling pathways exist to mediate flow/metabolism coupling in this unit, and these interact on multiple levels to ensure a coordinated blood flow response.

Disruption to neurovascular coupling is a common process that underlies many brain disease states (Girouard and Iadecola, 2006). Studies where nNOS is knocked out or inhibited have shown a decrease in neuronal activity related blood flow by up to 90% in the cerebellum (Akgoren et al., 1994), that is reversible in the presence of an exogenously administered NO donor (Piknova et al., 2011). nNOS inhibition in rats causes significant attenuation of the cerebral blood flow response to forepaw stimulation, which is disproportionate to the degree of attenuation of somatosensory evoked potentials (Stefanovic et al., 2007), indicating disruption to neurovascular coupling.

Metabolic pathways activated by glutamate also play a key role via activation of neuronal NMDA receptors (Attwell et al., 2010). This causes an influx of calcium that activates NOS, resulting in increased blood flow. Glutamate also stimulates prostaglandin and epoxyeicosatrienoic acid

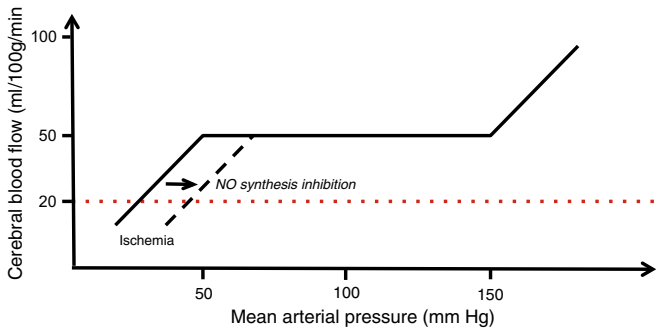

Fig. 1. Inhibition of NO synthesis results in right shift of hypotensive portion of cerebral autoregulatory curve. Cerebral autoregulation maintains an adequate and stable cerebral blood flow in the face of changes in mean arterial pressure (flat portion of the curve). The brain becomes vulnerable to ischaemia when cerebral blood flow falls below 20 ml/100 g/min (red dashed line). NOS inhibition results in right shift of the hypotensive portion of the autoregulatory curve, resulting in the brain becoming susceptible to ischaemia at higher perfusion pressures. Therefore NO signalling is essential in maintaining adequate cerebral blood flow under conditions of fluctuating mean arterial pressure.

(EET) synthesis in astrocytes via arachidonic acid (AA) pathways (Hamilton et al., 2010). This pathway can cause both vasoconstriction and vasodilatation (Golanov and Reis, 1994). Under conditions of NO depletion vasoconstriction is favoured via predominance of 20-hydroxyeicosatetraenoic acid (20-HETE) and decreased production of vasodilatory epoxyeicosatrienoic acids (EETs). These pathways are illustrated in Fig. 2.

NO and the cerebral blood flow response during hypoxia and hypercapnia

Hypoxia and hypercapnia are used as examples of physiological stresses as alterations in tissue partial pressures of oxygen and carbon dioxide are extremely common in critical illness following a brain insult (Peers et al., 2007). The NO signalling pathway plays a central role in the cerebral blood flow response to these alterations. NO pathway dysfunction occurs after brain tissue ischaemia, causing dysregulation of these normal physiological responses.

Hypoxia

Decreased arterial oxygen supply (hypoxia) increases the baseline level of CBF in humans (Mintun et al., 2001). NO plays a role in both signalling this event and the CBF response (Ho et al., 2012). Nonselective NOS inhibition abolishes the increased CBF response to hypoxia in rats (Takuwa et al., 2010). The evidence for this in humans is more indirect, but it has been demonstrated that exposure to hypoxia during adaptation to high altitudes increases NO levels and high-altitude dwellers such as Tibetans have increased circulating NO levels (Beall et al., 2012).

Hypercapnia

The modulation of cerebral vascular tone in response to changes in arterial partial pressure of carbon dioxide is known as chemoregulation, and this process may be viewed as one of the main regulators of cerebral vascular tone.

The cerebral vasodilatory response to hypercapnia is inhibited by L-arginine analogues, which act by blocking the NO synthesis pathway, and by NOS inhibitors (Wang et al., 1992; Kirkeby et al., 2000). Cerebral vasodilatation in response to hypercapnia is reduced after experimental traumatic brain injury in a rodent model and exogenous NO acts to reverse this (Zhang et al., 2002), helping to restore the normal physiological response.

A study on human cell cultures showed an increase in NO production in response to increased CO_2 levels (Fathi et al., 2011b). However, it is likely that the in vivo response differs from the in vitro response, due to multiple interactions of the NO pathway with other intracellular signalling pathways within the neurovascular unit.

Both hypoxia and hypercapnia are common after a cerebral insult due to decreased conscious levels that lead to hypoventilation. Therefore, in the face of a focal brain injury, NO depletion leads to disruption to the normal physiological response to alterations in oxygen and carbon dioxide, further exacerbating neuronal damage. Restoration of the normal adaptive response via replenishing cerebral NO levels may be efficacious at reducing secondary brain injury.

There is therefore a need for further investigation into the NO signalling pathway in a 'whole brain' model in humans, to precisely elucidate the specific mechanisms underlying the observed blood flow effects. Healthy volunteer studies examining the role for NO in the CBF responses to hypoxia and hypercapnia would enable clarification of the role of NO signalling during global disruptions to cerebral physiology, while studies in patients with cerebral injury would enable the effects of NO signalling during the evolution of focal brain pathology to be quantified.

The role of NO in the CBF response to disease

The NO signalling pathway influences the evolution of secondary brain injury in multiple ways, dependent on site, mechanism and timing of NO synthesis and concentration. NO has effects both on the vasculature and on a cellular level, and interacts with many other signalling pathways. This makes it difficult to translate results from animal models where conditions are tightly controlled to the patient population where the evolution of injury occurs unpredictably.

NO depletion

Relative NO depletion appears to occur in the early stages of cerebral injury regardless of the aetiology, e.g. after traumatic brain injury (TBI), subarachnoid haemorrhage (SAH), post-cardiac arrest and ischaemic stroke (Cherian et al., 2000; Tuzgen et al., 2003; Ahn et al., 2004). This occurs simultaneously with a reduction in blood flow (Hlatky et al.,

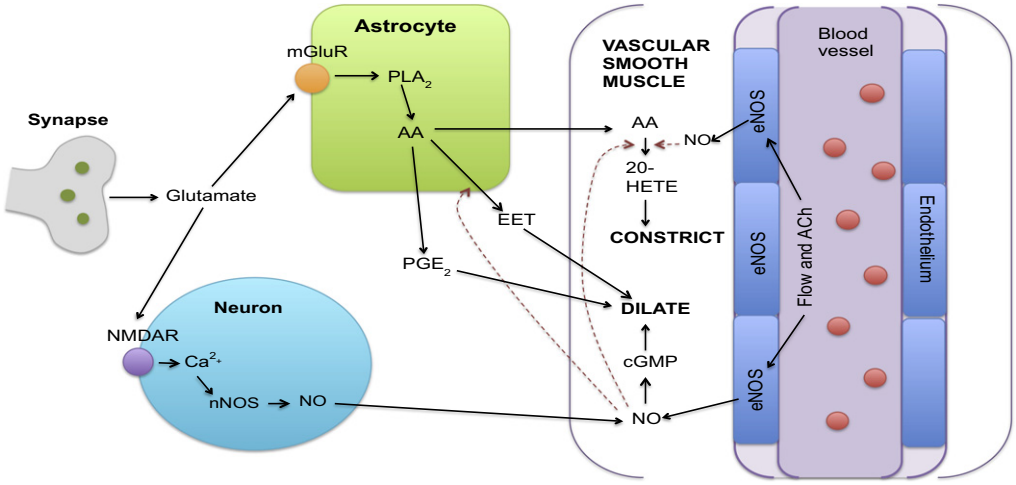

Fig. 2. Nitric oxide inhibits the production of key arachidonic acid-derived messengers. NO inhibits (dashed lines) the production of the vasoconstricting 20-HETE as well as the vasodilating EET. Endothelial nitric oxide synthase (eNOS) can be activated by flow-induced shear stress or by acetylcholine (ACh). This may be one mechanism whereby it may improve blood flow to the brain. NO = nitric oxide, NMDAR = NMDA receptor, PLA_2 = phospholipase A_2, nNOS = neuronal nitric oxide synthase, AA = arachidonic acid, mGLUr = metabotropic glutamate receptor, and PGE_2 = prostaglandin E_2.
Adapted with permission from Attwell et al. *Nature* 468(7321): 232–43.

2003). L-arginine, which enhances NO production, increases CBF (Cherian and Robertson, 2003; Lundblad and Bentzer, 2007). This indicates that disruption to NO signalling is part of a common pathophysiological pathway that leads to the development of secondary brain injury regardless of the mechanism of insult. NO depletion appears to be central in particular to many of the abnormal processes that occur after SAH.

SAH is usually caused by the rupture of a cerebral aneurysm located in the circle of Willis (van Gijn et al., 2007). The decrease in cerebrospinal fluid (CSF) NO metabolites is seen within 10 min of SAH and this is associated with vasoconstriction (Sehba et al., 2000). This is thought to be secondary to destruction of NOS function by haemoglobin (Tanishima, 1980; Reiter et al., 2002; Pluta, 2008). Decreased NO bioavailability is also caused by cerebral NO reacting with superoxide anions to produce peroxynitrite (ONOO) (Radi, 2013). Peroxynitrite formation may cause damage to mitochondria, the vascular endothelium and smooth muscle cells (Moro et al., 2005). In patients with SAH, increased CSF levels of the endogenous NOS synthase inhibitor asymmetric dimethylarginine (ADMA) were observed, and higher levels predicted poorer long term outcome (Li et al., 2014). However, another study showed a rise in CSF NO metabolites 24 h after SAH and the higher the level the worse the outcome (Ng et al., 2001). The discrepancies may be explained by the complexity surrounding the biochemical pathways relevant to the generation and elimination of NO and ADMA. An increase in NO metabolites is only an indirect measure of NO levels (the short half life of NO making it difficult to measure directly), and gives no information regarding the source or activity of the NO generated.

SAH is associated with microvascular constriction, resulting in microthrombus formation (Sabri et al., 2012) and microinfarctions (Stein et al., 2006). There is an associated global decrease in cerebral NO levels and a corresponding increase in cerebral P-selectin levels (Sabri et al., 2012). P-selectin is an endothelial cell adhesion molecule that promotes platelet aggregation and fibrin deposition (Zimmerman et al., 1992). The inhibition of NO synthesis results in increased P-selectin expression (Davenpeck et al., 1994), therefore NO depletion favours microthrombosis formation.

Cortical spreading depression (CSD) consists of a wave of electroencephalogram (EEG) silence moving across the cortical surface (Leao, 1944). CSDs play a role in both early brain injury (Nishizawa, 2013) and in delayed cerebral ischaemia after SAH (Dreier et al., 2006), as well as traumatic brain injury and stroke (Dreier, 2011). In healthy brain tissue, spreading depolarisation is associated with vasodilatation. However, in damaged brain tissue an inverse blood brain flow response occurs which results in vasoconstriction and tissue ischaemia (Dreier et al., 2013). Reduced levels of NO render the brain more susceptible to CSDs. Basal levels of NO determine the threshold of CSDs (Petzold et al., 2008) and NO also acts to restore impaired cerebrovascular reactivity after CSD (Scheckenbach et al., 2006).

Therefore it can be seen that NO signalling plays a crucial role in the blood flow changes that occur after subarachnoid haemorrhage. NO depletion plays a major role in early brain injury, microthrombus formation and cortical spreading depression. Targeting this pathway may enable partial restoration of physiological blood flow, helping to prevent secondary neuronal injury.

eNOS function

Impairment of eNOS activity is also implicated in many cellular mechanisms of neuronal injury (Srivastava et al., 2012) and is seen after SAH, TBI and ischaemic stroke. Innate eNOS activity determines susceptibility to injury, with possession of isoforms with greater activity conferring protection against secondary neuronal insult.

One of the major causes of morbidity and mortality following SAH is delayed cerebral ischaemia (DCI). This occurs in about 30% of patients (Weir and MacDonald, 1993; Khurana and Besser, 1997) typically between days 3 and 12 following SAH (Khurana and Besser, 1997). Originally this was thought to be caused by 'spasm' of cerebral arteries seen on CT angiography, but emerging evidence suggests that this is unlikely to be directly causal (Rowland et al., 2012). Recently it has been discovered that the NO signalling pathway plays a significant role in the pathogenesis of DCI (Vellimana et al., 2011). Genetic variation in the eNOS gene in humans influences the risk of DCI after SAH, as possession of an isoform that results in lower eNOS activity results in greater risk (Khurana et al., 2004; Starke et al., 2008).

eNOS activity is also important after TBI. The immunoreactivity of eNOS is increased in the area surrounding the contused area in the first 3 days after experimental trauma (Cobbs et al., 1997). eNOS knockout mice have greater reduction in CBF than wild type variants in the first 2 h after trauma (Lundblad et al., 2009). Genetic variants of eNOS influence the maintenance of CBF after severe TBI. As in SAH, patients with decreased endogenous eNOS levels had poorer outcomes than patients with alleles that did not affect eNOS levels (Robertson et al., 2011).

This is also the case in ischaemic stroke. Stroke is most often caused by a thrombotic or embolic blockage of a cerebral artery, causing interruption to blood flow, ischaemia and tissue death. eNOS deficient mice have bigger infarcts than wild type after middle cerebral artery occlusion in an animal model of ischaemic stroke (Huang et al., 1996).

eNOS uncoupling

Functional uncoupling of eNOS from its co-factor tetrahydrobiopterin (BH4) also occurs after cerebral insult. eNOS uncoupling occurs under ischaemic conditions, causing superoxide to be generated instead of NO (Vasquez-Vivar et al., 1998; Stuehr et al., 2001). Superoxide reacts with NO to form peroxynitrite, which is in itself a neurotoxin, decreasing levels of bioactive NO (Wink et al., 1993; Pluta et al., 2001). This reduction in NO bioavailability contributes further to secondary brain injury (Drexler and Hornig, 1999; Cai and Harrison, 2000).

eNOS uncoupling contributes to injury after SAH (Sabri et al., 2011a) and simvastatin acts to re-couple eNOS after SAH (Sabri et al., 2011b) in an experimental blood injection model of SAH in rodents. This implies that recoupling of eNOS may be a promising therapeutic target. However, a large multicentre randomised phase 3 trial failed to detect any benefit in the use of simvastatin for long- or short-term outcome in patients with aneurysmal SAH (Kirkpatrick et al., 2014). It is unclear why this was the case but it is likely that pathological aneurysm rupture triggers pathways that are not activated by a blood injection animal model, therefore statins may not be acting in the same fashion as in experimental models. Another possibility is that eNOS uncoupling has metabolic and cellular effects that affect other cellular signalling pathways, and the physiological differences between rodents and humans render results from animal studies difficult to interpret. For example, eNOS knockout appeared to confer neuroprotection in one study, indicating that the detrimental effects of eNOS uncoupling may outweigh the benefits of eNOS activity in this particular model (Sabri et al., 2013).

Ischaemia/reperfusion injury

This is a pathophysiological process responsible for organ injury in a variety of conditions such as myocardial infarction, organ transplantation and cardiac arrest. NO plays a pivotal role in the development of this condition, with NO donor agent administration reducing ischaemia/reperfusion (I/R) injury in animal models (Shiva et al., 2007). A systematic review of NO donor administration during animal stroke models demonstrated an overall improvement in cerebral blood flow and a decrease in infarction volume (Willmot et al., 2005). However, whether these results translate into the patient population with cerebral I/R injury (e.g. ischaemic stroke, cardiac arrest) has not yet been tested (Roberts et al., 2013) and represents an important area of future research. It is also likely that many of the neuroprotective effects of NO

donors are as a result of their metabolic and cellular effects and not simply the improvement of cerebral blood flow.

Cardiac arrest and subsequent recovery result in complete interruption followed by restoration of blood supply, and therefore may provide the best clinical model of ischaemia/reperfusion. Inhaled NO and intravenous sodium nitrite (a NO donor) have been shown to confer neuroprotection in mice and rats (Dezfulian et al., 2012; Ichinose, 2013), and this appears to be mediated by S-nitrosation of proteins rather than the canonical cyclic GMP signalling pathways (Dezfulian et al., 2012). eNOS mediated mechanisms are responsible for the neuroprotective effects of therapeutic hypothermia after cardiac arrest, and inhaled NO conferred neuroprotection in eNOS knockout mice, replicating the beneficial effects of hypothermia (Kida et al., 2014). Therefore, there is preliminary evidence for NO donors as a potential neurotherapeutic agent after cardiac arrest.

The role of iNOS

It has been thought that NO derived from iNOS is responsible for some of the neurotoxic actions of NO post-brain injury. One proposed mechanism by which this occurs is via the reaction of iNOS generated NO with superoxide generated from uncoupled eNOS to form peroxynitrite. The putative mechanism behind this is illustrated in Fig. 3.

However, this is an oversimplified view, and it is more likely that iNOS derived NO, like eNOS-NO, has several roles to play and that the balance between neurotoxicity and neuroprotection is dependent on many factors. As iNOS derived NO has multiple actions, results from animal studies may be focusing on its effects on cerebral blood flow (which is an easily measurable outcome) without accounting for the cellular and metabolic effects (which are more difficult to quantify).

NO derived from iNOS appears to contribute to neurotoxicity after ischaemic stroke, as animal knockout models have smaller infarcts than their wild type counterparts (Iadecola et al., 1997). An increase in NO above basal levels secondary to iNOS occurs 12–24 h after MCA occlusion and contributes to neurotoxicity, with iNOS inhibition leading to reduced infarct volume (Iadecola et al., 1995).

There is also evidence that NO produced by iNOS contributes to neurotoxicity following TBI. iNOS expression is significantly induced by brain injury, with peak concentrations occurring around 1–2 days after TBI (Clark et al., 1996; Wada et al., 1998). Patients with higher levels went on to have a worse outcome (Tisdall et al., 2013). A recent phase II clinical trial (NOSTRA) in TBI patients using the NOS inhibitor 4-amino-(6R,S)-5,6,7,8-tetrahydro-L-biopterin (VAS203), which preferentially inhibits iNOS (Terpolilli et al., 2009), showed significantly improved extended Glasgow Outcome Scores compared to placebo (Stover et al., 2014).

Data on the role of iNOS after TBI is conflicting. Attenuation of CBF at 72 h after TBI was demonstrated in iNOS knockout mice (Foley et al., 2008), which also correlated with long-term functional outcome (Sinz et al., 1999; Bayir et al., 2005). The inhibition of iNOS appeared to lead to exacerbation of deficits in cognitive performance and increased neuron loss (Sinz et al., 1999). However, these studies are confined to rodent models of controlled cortical impact injury, and the CBF results may be linked to pathological vasodilatation linked to inflammation. This may explain the apparent contradictory results shown, and emphasises that simply measuring cerebral blood flow does not give the entire picture with regard to the effects of NO in the brain after injury. Overall, the balance of evidence is in favour of iNOS activity being harmful in the patient population, and therefore inhibition of destructive cascades mediated via iNOS may have promise as a potential future therapy.

The role of NO signalling in the developing brain

Neonatal hypoxic–ischaemic insult is a major cause of acute mortality and is often associated with permanent neuropsychiatric problems, presenting a major public health concern. There is a lack of specific treatments besides hypothermia available for this condition; therefore identification and investigation of potential molecular therapeutic targets are of utmost importance.

NO is involved in several critical processes in the developing brain, including myelination (Olivier et al., 2010). It also plays a key role in the immature brain's response to hypoxic–ischaemic insult. Inhaled NO is neuroprotective in the developing brain, with reduction in the size of excitotoxic and ischaemic lesions in rats (Pansiot et al., 2010; Charriaut-Marlangue et al., 2012). Boosting NO-cGMP signalling using sildenafil, a phosphodiesterase type 5 inhibitor increases blood flow and has neuroprotective effects in the rat model of hypoxic–ischaemic brain injury (Charriaut-Marlangue et al., 2014).

However, as in the developed brain, it appears that dosage and timing of exposure to NO or molecules that influence the NO signalling pathway are extremely important. Inhaled NO given during the reperfusion period appears to have neurotoxic effects (Charriaut-Marlangue et al., 2012). This may be due to the activation of nNOS as inhibition leads to improved CBF and perfusion early after reoxygenation (Hsu et al., 2014). As in the developed brain, iNOS activation may be responsible for damage in the late (>24 h) phase (Higuchi et al., 1998). Therefore further work is needed to determine the optimal timing,

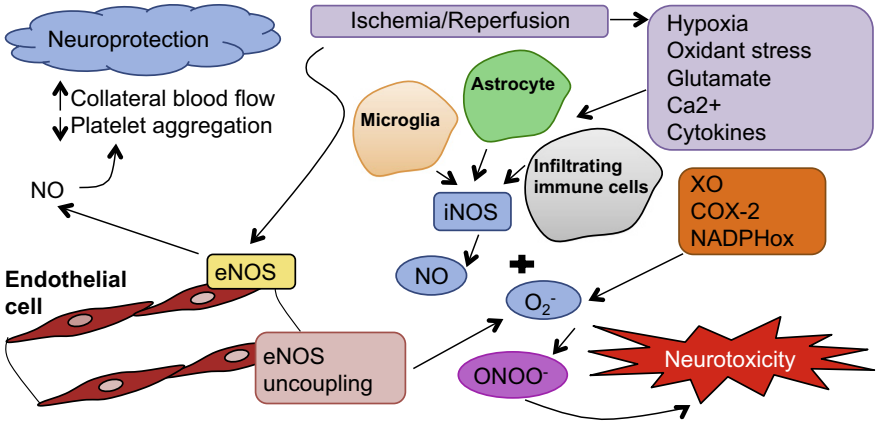

Fig. 3. Roles of NO and peroxynitrite in the pathophysiology of acute brain injury. Brain ischaemia and reperfusion leads to stimulation of endothelial NOS (eNOS), resulting in brief increases in endothelial NO generation, associated with neuroprotective actions. Ischaemic energy depletion and oxidant production trigger the release of glutamate, which results in stimulated activity inducible NOS (iNOS) in astrocytes, microglia and infiltrating inflammatory cells. iNOS activation also occurs in response to the release of inflammatory cytokines such as TNFα and IFNβ, and cellular signalling pathways responsible for the response to hypoxia. During the same period of time, superoxide production is enhanced due to uncoupling of eNOS, mitochondrial dysfunction, and the stimulated activity of NADPH oxidase, xanthine oxidase (XO), and cyclooxygenase-2 (COX-2). Formation of peroxynitrite is then favoured, damaging lipids, proteins and DNA that contributes significantly to neurotoxicity.
Adapted with permission from Pacher P et al. *Physiol Rev* 2007; 87:31.

Table 1
A selection of pharmacological studies detailing the effects of NO donors on animal models of brain injury.

Study	Model use	Type of agent	Dose	Outcomes
Terpolilli et al. (2013)	TBI; mice	Inhaled NO	50 ppm	Reduced lesion volume Improved neurological function
Terpolilli et al. (2012)	MCA occlusion; mice	Inhaled NO	5–50 ppm	Reduced infarct volume by 40%
Zhang et al. (1994)	MCA occlusion; rats	Intravenous SNP Intravenous SIN 1	3 mg/kg/h 1.5–6 mg/kg/h	Reduction in infarct size
Morikawa et al. (1994)	MCA occlusion; rats	Intravenous L-arginine	300 mg/kg	Reduced infarct volume by 35%
Jung et al. (2006)	MCA occlusion; rats	Intravenous sodium nitrite	48 and 480 nmol	33% and 77% reduction in infarct size Reduced neurological deficit
Li et al. (2013)	MCA occlusion; mice	Inhaled NO	10, 20, 40, 60 or 80 ppm	Dose and duration dependent reduction in infarct volume up to 50%
Fathi et al. (2011a)	Subarachnoid blood clot implantation; macaques	Intravenous sodium nitrite	300 μg/kg/h	26.9% reduction in vasospasm
Pluta et al. (2005)	Subarachnoid blood clot implantation; monkeys	Intravenous sodium nitrite	90 mg over 24 h	Prevention of vasospasm development

SNP = sodium nitroprusside.
SIN 1 = 3-morpholino-sydnonimine.

concentration and duration of NO therapy, and translation of these results to a human model is also needed.

Current therapies

Despite the controversies surrounding NO dysfunction after brain injury, there is animal evidence that increasing cerebral NO levels either directly using inhaled NO or indirectly using NO donors has neuroprotective effects. Table 1 summarises a selection of these pharmacological studies.

Future perspectives

Manipulation of the NO synthesis pathway shows great promise as a pharmacological therapeutic target. Exogenous NO appears to replicate the actions of eNOS derived NO, without contributing to neurotoxicity. NO donors may be neuroprotective by preventing NO depletion, helping to normalize capillary blood flow and improve oxygen delivery to the tissue (Shiva, 2013). Cellular mechanisms such as S-nitrosylation of proteins and effects on mitochondrial respiration also play an important role (Dezfulian et al., 2012).

However, the majority of this evidence has come from experimental animal models, largely focussing on ischaemic stroke whereas SAH, TBI and post-cardiac arrest are relatively under researched.

Cardiovascular instability when using classic NO donors such as glyceryl trinitrate (GTN) and sodium nitroprusside precludes their use in critically ill unstable patients, and makes results of drug trials difficult to interpret as local effects are negated by systemic blood pressure effects.

However, sodium nitrite is emerging as a feasible, safe NO donor with minimal effects on the systemic vasculature in patients after SAH (Pluta et al., 2011; Oldfield et al., 2013). It has also been used safely in cardiac arrest survivors (Dezfulian et al., 2012). This is because cerebral tissue is able to convert endogenous nitrite to NO without the need for oxygen as a substrate (van Faassen et al., 2009), meaning that when the tissues are hypoxic (such as after cerebral injury) nitrite is only converted to NO locally. The mechanisms underlying this are illustrated in Fig. 4.

The results from animal studies have been promising, with prevention and reversal of cerebral vasospasm after experimental SAH (Pluta et al., 2005; Fathi et al., 2011a). However, these are based on autologous blood clot injection models, and as discussed previously pathological aneurysm rupture is likely to activate multiple cellular signalling pathways that may not be replicated in these experimental models. Therefore large-scale clinical trials are needed to translate these results to patients.

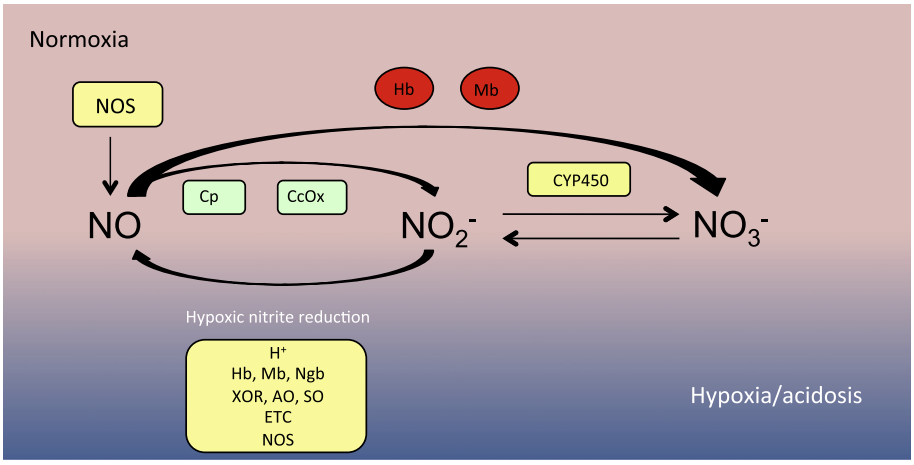

Fig. 4. The nitrate–NO cycle. In normoxia NOS is functional and generates NO, which is oxidised by haemoglobin (Hb) and myoglobin (Mb) to nitrate (NO_3^-) and by cytochrome c oxidase (ccox) and ceruloplasmin (Cp) to nitrite (NO_2^-). Nitrate is also derived from the diet as well as the normoxic oxidation of nitrite by cytochrome P450 enzymes (CYP450). In hypoxia, nitrite is reduced to bioactive NO by a number of mammalian nitrite reductase enzymes including Hb, Mb, neuroglobin (Ngb), xanthine oxidoreductase (XOR), aldehyde oxidase (AO), sulphite oxidase (SO), components of the mitochondrial electron transport chain (ETC.) and NOS.
Adapted with permission from Shiva S. *Redox Biol* 2013; 1(1): 40–44.

Conclusion

NO is an important mediator in the regulation of CBF in the resting state, acting as a key modulator on the pathways responsible for maintaining resting CBF and perfusion under physiological stresses such as fluctuations of blood pressure, hypoxia and hypercapnia. It also plays a key role in the development of the healthy brain. Disruption to NO synthesis and metabolism underlies many of the pathophysiological processes that occur after brain injury, and appears to occur regardless of the mode of injury. The available evidence suggests that NO derived from eNOS is neuroprotective after acquired brain injury whereas NO synthesised by iNOS contributes to further damage, and that this difference is due to differences in timing, spatial location and concentration of NO generated by each isoform. This has led to investigation of drugs that influence NO signalling as potential therapeutic agents. In particular, NO donors have been shown to be neuroprotective and efficacious in reducing secondary brain injury in animals, potentially by replicating some of the effects of endogenous NO derived from eNOS. iNOS inhibitors may also hold promise as a future neurotherapeutic target. Future studies should focus on investigating the therapeutic potential of the many other signalling molecules that interact with the NO synthesis pathway and determining long-term side effects of NO pathway manipulation in the injured brain. The hypotheses presented in this review must be tested in large-scale clinical trials to verify whether promising results from animal studies can translate into the patient population. Better understanding of the role of the NO pathway may lead to the development of exciting new pharmacotherapies designed to minimise secondary brain injury, with the aim of reducing the morbidity and mortality from devastating conditions such as subarachnoid haemorrhage, ischaemic stroke, traumatic brain injury and neonatal hypoxic–ischaemic insult.

List of abbreviations

ADMA	asymmetric dimethylarginine
CO_2	carbon dioxide
CBF	cerebral blood flow
CPP	cerebral perfusion pressure
CSF	cerebrospinal fluid
CT	computerised tomography
CSD	cortical spreading depression
cGMP	cyclic guanosine monophosphate
20-HETE	20-hydroxyeicosatetraenoic acid
EEG	electroencephalogram
DCI	delayed cerebral ischaemia
eNOS	endothelial nitric oxide synthase
EET	epoxyeicosatrienoic acid
GTN	glyceryl tri nitrate
iNOS	inducible nitric oxide synthase
I/R	ischaemia/reperfusion
MCA	middle cerebral artery
NMDA	N-methyl D-aspartic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
ONOO	peroxynitrite
SIN 1	3-morpholino-sydnonimine
SNP	sodium nitroprusside
SAH	subarachnoid haemorrhage
TBI	traumatic brain injury

Details of authors' contributions

All authors contributed to the conception, writing, critical review and revision of the manuscript.

Declaration of interests

We declare no competing interests.

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