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REVIEW ARTICLE

Nitric oxide and the brain. Part 1: Mechanisms of regulation, transport and effects on the developing brain

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Apart from its known actions as a pulmonary vasodilator, nitric oxide (NO) is a key signal mediator in the neonatal brain. Despite the extensive use of NO for pulmonary artery hypertension (PAH), its actions in the setting of brain hypoxia and ischemia, which co-exists with PAH in 20–30% of affected infants, are not well established. This review focuses on the mechanisms of actions of NO covering the basic, translational, and clinical evidence of its neuroprotective and neurotoxic properties. In this first part, we present the physiology of transport and delivery of NO to the brain and the regulation of cerebrovascular and systemic circulation by NO, as well the role of NO in the development of the immature brain.

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IMPACT:

- NO can be transferred from the site of production to the site of action rapidly and affects the central nervous system.
- Inhaled NO (iNO), a commonly used medication, can have significant effects on the neonatal brain.
- NO regulates the cerebrovascular and systemic circulation and plays a role in the development of the immature brain.
- This review describes the properties of NO under physiologic conditions and under stress.
- The impact of this review is that it describes the effects of NO, especially regarding the vulnerable neonatal brain, and helps understand the conditions that could contribute to neurotoxicity or neuroprotection.

INTRODUCTION

Nitric oxide (NO) is produced endogenously by a variety of cells following the reaction of the amino acid L-arginine with molecular oxygen with production of L-citrulline by NO synthases (NOS). There are three types of NOS that produce endogenous NO in the brain: neuronal NOS (nNOS, or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The enzyme eNOS is a membrane-bound isoform found in endothelial cells, and nNOS is a cytosolic isoform first described in neuronal tissues. All of these isoforms are activated by intracellular calcium-dependent binding of calmodulin (CaM), whereas iNOS is inducible in a variety of cells including endothelial and innate immune cells.[1]

One of the key intracellular effects of NO involves the production of cyclic GMP (cGMP), which is responsible for its biological actions. Because of its vasodilatory properties, inhaled NO (iNO) has been used for the treatment of pulmonary artery hypertension,[2–6] an intervention that has decreased the need for extracorporeal membrane oxygenation.[7,8] Indeed, iNO improves cardiovascular function in sepsis,[9,10] and after ischemia,[11] facilitates blood flow to ischemic tissues,[12] improves renal function,[13] increases the oxygen affinity of sickle cell erythrocytes,[14] improves mesenteric flow,[15] decreases trypsinogen activation peptides in pancreatitis,[16] and has a significant impact in hemopoietic tissues, including platelets, neutrophils, and platelet–leukocyte interactions.[17–19] In the brain, NO plays an important role as a biochemical neurotransmitter. On the other hand, NO is also associated with direct neurotoxic effects, which are attributed in part to energy failure, lipid peroxidation, increased production of peroxynitrite, and protein nitrosylation.[20–22] The underlying mechanisms of NO transport and the effects on brain function are the subject of this review.

MECHANISMS OF REGULATION AND TRANSPORT OF NITRIC OXIDE

In order to prove an effect of iNO arising from the primary organ of action—the lung, to a distal organ—the brain, it is important to determine the mechanisms of biological interactions and transport of NO from one site to the other. The extracellular fate of iNO is hindered by its short half-life, which is estimated to be <5 s, thereby necessitating a transport mechanism to influence more distal sites. NO transport involves the formation of complexes with proteins into nitrosothiols (usually termed as R-SNO). The formation of R-SNO occurs via mechanisms that involve the reaction of NO with iron that is bound to proteins. The most abundant of these proteins is hemoglobin (Hgb). Hgb contains both iron and a thiol group and is therefore ideal for transport and regulation of NO.[23,24] *S*-nitrosothiol-modified Hgb (SNO-Hgb) or NO-related metabolites account for the distant transfer of NO and explain its extrapulmonary effects. NO blood concentrations and its transport are regulated via complex mechanisms, which involve key allosteric interactions with Hgb and oxygen (O_2). Heme concentrations in blood are ~2 mM, while those of NO are <100 nM.[23,25] NO production rates cannot explain the NO concentrations found in plasma where there are always unoccupied heme sites, even in the arterial circulation. As a result, Hgb cannot be viewed just as a

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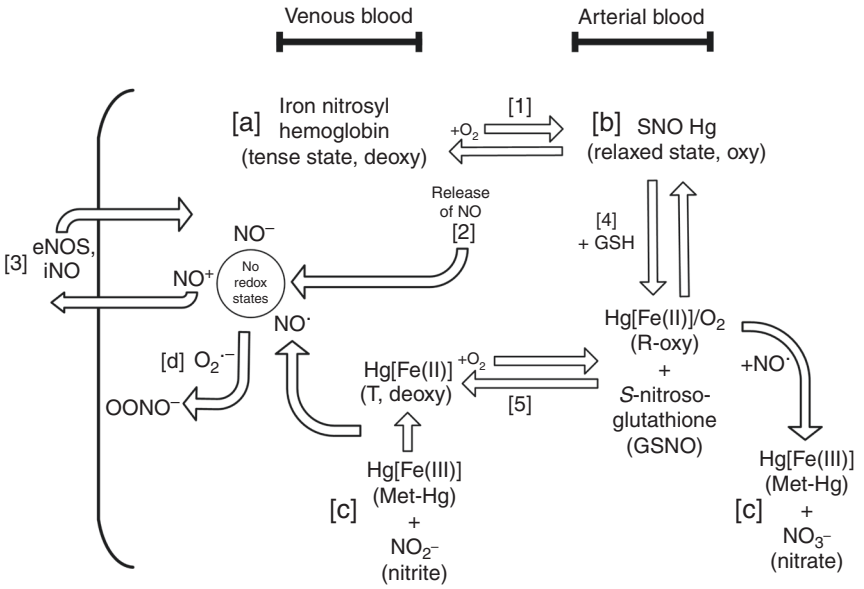

Fig. 1 Cellular interactions of NO. The presence of molecular oxygen and other factors such as CO_2 contribute to the allosteric conversion of Hgb from a tense deoxygenated state to the relaxed oxygenated form. Oxygen also serves as electron donor to convert the iron-nitrosyl hemoglobin to *S*-nitroso-hemoglobin, SNO-Hgb (product [b], Reaction [1]). In lungs exposed to high oxygen tension Hgb is *S*-nitrosylated with a simultaneous decline of iron-nitrosyl hemoglobin (product [a]). This can occur by intramolecular transport of NO to cysteine (Cysβ93). NO is released by the cysteine residue when red blood cell is under conditions of deoxygenation (Reactions [2] and [3]). SNO-Hgb is also in balance with other *S*-nitroso-thiols such as glutathione (GSH). High concentrations of glutathione shift the reaction towards the deoxy (Tense) form (Reactions [4] and [5]) providing an endogenous protective mechanism for NO. Reaction [c] shows alternative ways of production or consumption of NO in different redox states of the cell, either with production of Met-Hg and nitrate or by consumption of nitrite and Met-Hg. Reaction [d] shows the production of peroxynitrite upon reaction with superoxide. Glutathione as well as the product of Reaction [4], *S*-nitroso-glutathione (GSNO), exacerbates the vasodilatory effect of SNO-Hgb. In SNO-Hgb, NO may exert its actions via nitrosonium ion (NO^+) rather than the free radical NO. Nitrosonium is created by the reaction of NO with molecules that carry a metal center such as hemoglobin or ceruloplasmin, typically with the formation of intermediate thiols, which can create SNO if there is an electron acceptor such as oxygen, adequate flavin adenine dinucleotide (FAD), or nicotinamide adenine dinucleotide (NAD). NO is unlikely to be able to escape the RBCs as a free radical and is likely released as nitrosonium via the formation of iron-nitrosyl Hg as intermediate or as SNO-Hgb (Reaction [2]).

reservoir for NO.[23,25,26] The physiologic transport of NO via Hgb was first described by Stamler and co-workers[23] in 1996 who identified that NO binds at a highly conserved cysteine residue (β93 position) to form SNO-Hgb.[27] Gow and Stamler[27] and Stamler and co-workers[28] suggested that the actions of Hgb in terms of NO regulation vary depending on context with delivery, storage, or consumption of NO.

NO transport and availability depend on changes of Hgb between "relaxed" and "tense" states and the presence of a "thermodynamic" linkage.[29] SNO-Hgb is the predominant form in oxygenated arterial blood, while iron-nitrosyl-Hgb is higher in venous blood. In Fig. 1, we present the complex interactions of NO, including (a) the reaction with the iron of the alpha subunits of deoxy-Hgb in red blood cells (RBCs) to form iron-nitrosyl-Hgb, (b) the reaction with cysteine resulting in the formation of SNO-Hgb, (c) its oxidation to nitrate (NO_3^-) contributing to the formation of the ferric form of Hgb (Met Hgb, Hgb[Fe(III)]); this reaction is not significant under physiologic conditions since Met Hgb can be recycled back to deoxy-Hgb by intra-erythrocyte Hgb reductase with simultaneous production of NO and consumption of nitrite,[27] and (d) the reaction of NO with superoxide ($O_2^{\cdot}-$) to form peroxynitrite ($OONO^-$). Under physiologic conditions, the formation of peroxynitrite is limited because the superoxide dismutase (SOD)/catalase system is usually able to limit the concentration of the superoxide. Any increase in the production of superoxide, as happens in metabolic stress, or if SOD is decreased or deactivated, can result in the production of peroxynitrite. The interactions described in (a) and (b) represent the predominant forms that exist and contribute to transport and biological actions of NO, while (c) and (d) are related to NO toxicity. A key intermediate contributing to the protective role of NO in the brain is the formation of *S*-nitroso-glutathione (GSNO) (Fig. 1, [4]). GSNO is 100 times more potent free radical scavenger than glutathione itself,[30] operates as an endogenous NO reservoir, and protects against oxidative stress.[31,32] Using these mechanisms, NO protects the brain from lipid peroxidation by scavenging lipid peroxyl radicals. GSNO can also neutralize peroxynitrite, which explains the weak oxidative effects of peroxynitrite in the brain in vivo and in vitro.[33,34]

Both iron-nitrosyl-Hgb and SNO-Hgb concentrations have been measured in the circulation.[23,24,35] In umbilical cord blood samples that were obtained from newborns delivered between 37 and 42 weeks of gestation, SNO-Hgb was almost twice as high in the umbilical vein than in the artery.[36] NO in the blood activates soluble guanylyl cyclase in vascular smooth muscle to maintain normal vascular tone and oxygen delivery, whereas excess NO is sequestered by Hgb and could contribute to more effective distal delivery of O_2 and NO.[37]

The most likely source of NO that is physiologically transferred via the RBCs is eNOS, although inhaled NO is another possibility (Fig. 1, [3]). As RBCs pass through lung capillaries in close proximity to alveoli, both NO and O_2 are taken up. Because of the high tension of O_2, the allosteric conformation of Hgb changes and NO is taken up to form SNO-Hgb. In peripheral tissues, RBC oxygen falls as O_2 is delivered, causing conformational change to iron-nitrosyl Hgb and NO is released, resulting in regional vasodilatation so as to match its function with the metabolic demand of the tissue.

NO exists in different redox forms that are derived from the oxidation states of nitrogen and mimic those of oxygen: free radical $NO^{\cdot}$, nitrosonium NO^+, and nitroxyl anion NO^-. Of these, nitrosonium ion contributes to the formation of SNOs by being a

potent nitrosating agent. Nitrosonium is created by the reaction of NO with molecules that carry a metal center such as Hgb or ceruloplasmin,[27,38] with the formation of intermediate thiols that can create SNO if there is an electron acceptor such as oxygen with adequate flavin adenine dinucleotide and nicotinamide adenine dinucleotide.[39] NO is unlikely to be able to escape the RBCs as a free radical and is speculated to be released as nitrosonium via the formation of iron-nitrosyl Hgb as an intermediate or as SNO-Hgb (Fig. 1, [2]).[40,41]

The transport of NO as SNO-Hgb from the RBC to the site of action occurs using two mechanisms (a) as thiol carriers (glutathione disulfide-stimulated ATPases), which are exported by the RBCs,[42] or (b) by direct association of Hgb with the red cell membrane, which will be able to donate the NO group from Cysβ93.[43]

The local production of NO in the brain has multiple possible sources. The obvious site of production, the endothelium, appears to contribute only partially as shown in studies of arteries with denuded endothelium[44] or destruction of the endothelium with different methods such as light-dye techniques.[45] NO most likely also arises from sites where NOS has been identified, such as neurons, astrocytes, and perivascular nerves.[46] As noted earlier, another stimulus for intravascular NO production is the shear stress from blood flow, particularly in smaller arteries.[47,48]

NO AND CEREBROVASCULAR AUTOREGULATION

(A) *NOS effect*: Cerebral autoregulation involves complex interactions between neurogenic and endothelial factors with the goal to match cerebral blood flow (CBF) to metabolic demand. The role of endogenous NO in the maintenance of CBF has been investigated using indirect approaches such as by blocking NOS with pharmacologic inhibitors. Two commonly used inhibitors of NOS are L-arginine methyl ester (L-NAME) and L-monomethyl L-arginine, which are analogs of L-arginine and interfere with the active site of the enzymes. NOS inhibitors have several serious limitations, including their interference of cytochrome C, their ability to induce apoptosis, as well as the ability for the de novo production of NO.[49,50]

Under normal conditions, NOS inhibition reduces CBF.[51,52] After brain hypoxia/ischemia and reperfusion, both nNOS and eNOS participate in the modulation of CBF. In Table 1a, we summarize data from key animal studies that show the role of NOS under normal and various abnormal conditions. Basal microvascular tone is controlled by NO produced by nNOS localized in nerve endings in close proximity to the vascular system. While nNOS is involved in the regulation of cerebral vascular tone and blood flow, particularly in response to hypoxia and hypotension, eNOS regulates flow-mediated vasodilatation.

After hypoxia, NO content rises as a result of increasing calcium concentrations. In the acute phase of ischemia, NO is produced by the activation of nNOS, while later microglia-related eNOS or iNOS (neuro-inflammation) also contribute to the production of NO.[53,54] In a model of transient focal cerebral ischemia in rats, the effect of the low versus high doses and timing of administration of L-NAME was studied. Interestingly, mild decrease in the ability of the brain to produce NO is neuroprotective following brief ischemia, as well as in stroke.[55,56] In acute ischemia, NO is a major mediator of deviation of CB via collaterals to areas of brain that have been subjected to ischemia.

The severity of hypoxemia might also affect the role of NO as a mediator of CBF. Experiments in rats using microspheres noted that CBF increased after a drop in PaO_2 to 33 mmHg (severe hypoxemia) or to 45–60 mmHg (moderate hypoxemia). The increase in CBF in the severely hypoxic group was attenuated when NOS was blocked with L-NAME.[51,57] Despite conflicting results, partial blockade of the NOS system is neuroprotective, while complete inhibition is destructive.[58–61]

(B) *iNO effect*: In Table 1b, key studies and their associated methods in various animal models are presented. Of note, iNO did not significantly affect the CBF in a ventilated sheep model when measured by microsphere technique.[62] On the other hand, a dose-dependent increase in CBF was observed in a swine model under physiologic conditions without affecting systemic blood pressure. CBF in the latter study was assessed with NIRS and indicator dilution techniques.[63] Given the differences in methods, animal models and developmental stages, the effect of iNO on the distal versus proximal cerebral vessels needs to be investigated further.

Initiation of 20 p.p.m. of iNO is associated with a significant and time-related increase in brain NO as measured with continuous monitoring via a voltametric technique with carbon fiber electrodes.[64] Investigators noted that if iNO was applied during the ischemic phase, it was protective by increasing collateral arterial recruitment, increasing blood flow to the ischemic brain and decreasing oxidative injury as measured by nitrotyrosine-positive brain cells. However, if iNO was applied during reperfusion, the effects were detrimental as shown by increasing infarct size and blood flow. In this experiment, 20 p.p.m. of iNO was able to overcome NOS inhibition with L-NAME.[65] In mice, iNO applied in 30% oxygen/70% air mixture formed nitric oxide carriers in the blood that distributed throughout the body. After experimental cerebral ischemia induced by transient middle cerebral artery occlusion, iNO was associated with dilated arterioles, increased collateral blood flow, and reduced ischemic brain damage, improving the neurological outcome.[66] NOS inhibitors impair cerebrovascular autoregulation during moderate hypotension in rats. This phenomenon was observed in the cerebrum, cerebellar cortex, and basal ganglia.[67] NO also attenuates the normal oscillations of the diameter of the cerebral arterioles, a reaction opposite to that observed in the mesenteric arteries.[68]

The stage of development and the duration, as well as the dose of iNO also influence outcomes. Thus, iNO at 40 p.p.m. for 23 h administered 1 h after cardiac arrest and cardiopulmonary resuscitation (CPR) in adult mice prevents water diffusion abnormalities, caspase-3 activation, and cytokine induction in the brain. Deficiency of the α1 subunit of soluble guanyl cyclase prevented the ability of iNO to improve outcomes after CPR.[69] In these studies, the application of iNO at higher dose and for a prolonged time during the reperfusion phase was associated with positive effects. Further, using a modified Vannucci model in PN9 mice, high dose iNO (50 p.p.m.) reduced neuronal damage when given during hypoxia,[66] while iNO given after hypoxia–ischemia increased ischemic lesions.[70] iNO at a higher dose (80 p.p.m.) was associated with increased blood flow and deleterious effects, which could be related to the accumulation of peroxynitrite.[65]

The biochemical effects of iNO that contribute to the maintenance of brain autoregulation are poorly investigated and most of the knowledge is extrapolated from studies that involve the endogenous production of NO as noted above. Central nervous system (CNS) vascular tone is mediated by control of endogenous cellular mechanisms, such as local cGMP concentration. Indeed, cGMP along with other factors, such as prostaglandin E, cyclic AMP, and protein kinase A, can affect the activation of ATP-sensitive (K_{ATP}) and voltage-gated (K_V) K^+ channels, which exist at the cell membrane and, once activated, can cause hyperpolarization of the cell, membrane calcium channel deactivation, decrease in intracellular calcium, and vasodilatation. Similar effects can occur via direct decrease in calcium release from sarco/endoplasmic reticulum Ca^{2+}-ATPase via the protein kinase G. Brain injury and associated loss of autoregulation involves the deactivation of the these K^+ channels and iNO modulates their function to be protective in maintaining autoregulation.[71] Although the exact mechanisms have not been elucidated, one effect of iNO is to interfere with the production of endothelin-1 (ET-1) and activation of extracellular signal-regulated kinase (ERK) isoform of mitogen-activated protein kinase (MAPK).[72] ET-1 is

Table 1. (a) NOS system and autoregulation and (b) iNO and brain (CBF, hypoxia–ischemia).

(a) NOS system and autoregulation

Hypothesis procedures	Experimental conditions	Types of NOS investigated	Results	Ref.
Inhibition of NOS in a P7 rats, brain ischemia, measurement of flow in the brain arterial system	Brain ischemia: L-middle cerebral artery occlusion, followed with temporary, for 50 min, both common carotid occlusions	Both specific (eNOS and nNOS) and non-specific NOS inhibition (global)	Global inhibition: increased cortical ischemia. eNOS inhibition: increased CBF and increased infarct only in males. Lower phosphorylation of nNOS at Ser (847) in males and increased in females at 24 h. nNOS inhibition also increased CBF only in males	[60]
Male Sprague and Dawley rats, samples from basal forebrain and cortex	Under normal conditions. Immunochemistry and electron microscopy of basal forebrain neurons for nNOS	nNOS	Basal forebrain NOS neurons provide ~1/3 of cortical NOS innervation. At electron microscopy: most of the NOS corresponded to nerve terminal, which were mainly in close vicinity with the blood vessels	[95]
Sprague and Dawley rats anesthetized, intubated, and ventilated. Micro-electrodes placed in brain to assess NO, perivascular O_2	Induced hypotension, decreased paO_2, and assessment of NO responses, also activated nNOS with glutamate via NMDA	nNOS vs. eNOS effect and relative contributions in NO production	nNOS predominantly increased NO during decreased periarteriolar paO_2 (e.g., in hypotension), while eNOS was the dominant source of NO for flow shear mechanisms	[96]
Homozygous eNOS mutant mice, generated by homologous recombination	The thoracic aorta was dissected. Endothelium-dependent relaxation of aortic rings in response to acetylcholine	eNOS	eNOS-mediated basal vasodilation. eNOS mutant mice show a decrease in blood pressure in response to L-NA. Non-endothelial isoforms of NOS may be involved in maintaining blood pressure	[97]
Assessed the endothelial constitutive nitric oxide synthase (ecNOS) in the developing rat brain	Immunostaining patterns of a monoclonal antibody against ecNOS	eNOS and its distribution	ecNOS is involved in the embryonic angiogenesis and the regulation of hemodynamic functions of brain vasculature throughout the individual life	[98]
eNOS knockout mice with S1179DeNOS	Assessment of endothelium-dependent vasodilatation in pressurized carotid arteries	eNOS	Transduction of the endothelium of carotid arteries from eNOS knockout mice with S1179DeNOS completely restored NO-mediated dilatation to acetylcholine (ACh)	[99]
CBF changes were monitored, using laser Doppler flowmetry, in rats subjected to 30 min of forebrain ischemia	Investigated relative contributions from nNOS and eNOS in their ability to modulate CBF changes, in the hippocampus and striatum after ischemia	nNOS and eNOS	nNOS contribution to intra-ischemic vasodilation was substantially greater than eNOS. In the hippocampus, there was higher intra-ischemic CBF. This was associated to a low eNOS contribution on vascular function in that structure and CBF redistribution to the hippocampus when eNOS activity is blocked globally	[100]
Female and male neuronal nitric oxide synthase (nNOS) knockout (nNOS−/−) mice examined in terms of histological injury after middle cerebral artery occlusion (MCAO) relative to wild type (WT)	Tested the hypothesis that ischemic cell death from neuronally NO or poly-ADP ribose polymerase (PARP-1) activation is affected by sex	nNOS in association with PARP-1 as a cause of differences between sexes in mice	Female neuronal nitric oxide synthase (nNOS) knockout (nNOS−/−) mice exhibited exacerbated histological injury after middle cerebral artery occlusion (MCAO) relative to wild-type (WT) females, unlike the protection observed in male nNOS−/− littermates. Similarly, treatment with the nNOS inhibitor (7-nitroindozole, 25 mg/kg) increased infarction in female C57Bl6 WT mice, but protected male mice.	[101]

(b) iNO and brain (CBF, hypoxia–ischemia)

Condition	Dose of iNO	Species	Effect	Ref.
Normal	5, 10, and 50 p.p.m.	Anesthetized pigs	iNO increased cerebral blood volume significantly and reversibly. Cerebral transit time increased, while cerebral blood flow remained unchanged, which thought to be explained by a predominant vasodilatory action of iNO in the venous compartment	[63]

Table 1. continued

(b) iNO and brain (CBF, hypoxia–ischemia)

Condition	Dose of iNO	Species	Effect	Ref.
Hypoxia–ischemia	20 p.p.m.	P7 rat	iNO (at 20 p.p.m.) is transported to the brain and capable of increasing blood flow in the great arteries after the blockade of NO synthesis. iNO can replace intravascular NO to maintain normal vascular function	65
Hypoxia–ischemia	80 p.p.m.	P7 rat	Both 80 p.p.m. iNO given during ischemia and 5 or 20 p.p.m. iNO given 30 min after reperfusion were associated with increased brain damage	65
Cardiac arrest and cardiopulmonary resuscitation (CPR)	40 p.p.m.	Adult mice	Beneficial effect if iNO was started 1 h after CPR. Reduced water diffusion abnormalities and neuronal damage, as well improved survival	69
Transient focal cerebral ischemia secondary to traumatic brain injury	40 p.p.m.	Male C57Bl/6 mice	iNO significantly improved CBF and reduced intracranial pressure after TBI. Long-term application (24 h NO inhalation) resulted in reduced lesion volume, reduced brain edema formation, and less blood–brain barrier disruption, as well as improved neurological function	102
Hypoxia–ischemia	5 p.p.m.	Newborn rats	iNO was associated with decreased astrogliosis, microglial activation, and apoptotic cell death, increased density of proliferating preoligodendrocytes and enhanced oligodendroglia maturation. iNO was associated with the upregulation of both transcription and neurotrophic factors: P27kip1 (an oligodendrocyte differentiation factor), Sox10, and BDNF	103
Hypoxia–ischemia	20 p.p.m.	P5 rat pups	iNO is neuroprotective against excitotoxic brain damage, via pCREB and downregulation of several subunits of NMDA receptors	76
Normal, hypoxemia and hyperoxia with the addition of iNO	20 p.p.m.	Fetal sheep	Left ventricular cardiac output (LV CO) and CBF were measured with radiolabeled microspheres. Cerebral oxygen delivery and consumption were calculated using measurements of arterial and cerebral venous (sagittal sinus) oxygen content. Acute pulmonary vasodilation caused by iNO did not affect LV CO, CBF, or cerebral oxygen consumption	62

known to impair opening of K^+ channels via the release of ROS and phosphorylation of ERK/MAPK, a distal signaling system important in the control of CBF.

In summary, the preclinical studies highlighted above show a benefit in using iNO during the acute phase of brain ischemia in contrast to its use during the reperfusion period, which might exacerbate brain injury.

ROLE OF INO IN NEURONAL DEVELOPMENT, MYELINATION, AND MEMORY

Endogenously produced NO plays an important role in the development of the CNS.[73,74] NO promotes immature oligodendrocyte myelination and in animal models of stroke is neuroprotective,[65,75–77] possibly by changing the expression patterns of semaphorins, key membrane proteins for axonal growth guidance and migration.[75,78] The global inhibition of NOS in rodents significantly decreases myelin density in the corpus callosum and striatum and affects long-term cognitive behavior.[75] Gibbs et al.[79] showed that NO works to coordinate proliferation and patterning during brain development and endogenous NO production is upregulated after brain damage which results in neurogenesis.[80,81]

Olivier et al.[75] showed that iNO exposure at 20 p.p.m. during the first week of life was associated with significant increase in myelin fiber density at P7 in several white matter areas, including the lateral corpus callosum in the normal neonatal rat. The effect was more pronounced in periventricular white matter and to a lesser degree in the cortex. In P1 animals, brain cGMP was increased within 2 h of initiation of iNO, suggesting that the effects of iNO were observed in the brain. In P3 and P7 animals, the effect was less obvious, suggesting that there is a change in response with age.[75] Rodents who were treated with low dose iNO (5 p.p.m.) demonstrated attenuated hyperoxia-induced white matter inflammation, cell death, and enhanced the density of proliferating oligodendrocytes and oligodendroglia maturation. iNO enhanced an early upregulation of P27kip1 and brain-derived growth factor, and iNO-treated animals maintained learning scores to a level similar to that of normoxic controls.[82] Phan Duy et al.[83] showed that both early (30 h) and late (7 days) administration of iNO (at 20 p.p.m.) caused proliferative effects on progenitor cells on several zones of the subventricular zone, white matter, and cortex. In experiments that included co-labeling of progenitor cells with the proliferative marker BrDu and caspase-3, a marker of apoptosis, iNO prevented apoptosis and these cells had normal survival. Under physiologic conditions, astrocytes as well as pericytes were increased in cortical and white matter areas, changes suggestive of increased angiogenesis. iNO could involve the modulation of erythropoietin (EPO) which was associated with enhancement in the proliferation and migration of the site of injury of subventricular zone (SVZ) neural progenitors.

The effects of iNO on the development and organization of neuronal pathways, suggested possible effects on memory. NOS inhibitors suppress spatial memory,[84] object recognition,[85] and alter the behavior in a variety of animal models.[86,87] It is interesting that nNOS appears to be more specific for the development of spatial and working memory,[88] social interactions, and fear,[89] while eNOS knockout mice exhibit enhanced spatial learning, but increased anxiety-like behaviors.[90] Both short- and long-term memory have been studied in a variety of animal models of traumatic brain injury (TBI). Typically, after TBI, there is an inflammatory response with activation of microglia and astrocytes with secondary increases in interleukins followed by cerebral edema, loss of autoregulation, and eventually neuronal loss. It is interesting that there is a relative decrease in brain NO between 5 min to 3 h after TBI, which makes the need of supplementing iNO under these conditions a potential therapeutic intervention. For example, in a study where mice were given mild TBI, exposure to iNO for 4, 8, and 24 h at 10 p.p.m., was associated with improved short-term memory (as measured by Object Recognition Task Assessment) at 1, 3, and 7 days post injury.[91] By histology, NO-treated mice had an improved activation of microglia (as assessed by CD45) and astrocytes (by glial fibrillary acidic protein assays), and this effect appeared to improve with the duration of treatment up to 8 h, but not for longer exposures.[92] The effects of iNO in modulation of long-term memory are less well described.[93,94]

These preclinical studies demonstrate that iNO at lower doses improves myelination and neuronal density, as well as associated memory functions.

In summary, in this first of a two-part review, we describe the cellular and molecular mechanisms of NO in the CNS with an effort to determine potential neuroprotective or neurotoxic effects of its inhaled form (iNO), a commonly used medication in the neonatal intensive care unit.

iNO has profound local vasodilatory effects in the lung and understanding the mechanism of transport and unloading of NO by the RBC is important, especially since it affects brain autoregulation, metabolism, and function. Despite the extensive use of iNO as a pulmonary vasodilator, the exact effects in the neonatal brain remain poorly investigated.

NO functions in the CNS like a neurotransmitter with unique and distinguishing features. Some of these features such as the ability for fast production, the multiple sources of production, and its ability to move extremely fast through biological membranes away from its production site, differ from other locally produced neurotransmitters. Despite its short half-life, NO can affect other downstream pathways, modulating their action and hence causing long-lasting biological effects.

The inhaled form of NO contributes to neuroprotection after ischemia in the developing brain depending on the concentrations, stage of development, the timing, and the duration of exposure after the insult. Excessive NO is associated with free radical production and neurotoxicity. The neonatal brain is more susceptible to oxidative stress after hypoxia–ischemia and more sensitive to downstream activation of apoptotic mechanisms. NO can interfere directly at the level of mitochondria and trigger the initiation of apoptosis. Although preclinical studies summarized in this review suggest a benefit in the acute ischemic phase, there are also potential detrimental effects during reperfusion. iNO at low doses improves myelination and neuronal density, as well as associated functions such as memory, while higher doses result in toxicity.

Significant gaps in knowledge, due to lack of studies especially in newborns, include methods capable of measuring brain NO concentrations in real time and their correlation with anatomic sites as well as NOS subtype activity (nNOS, eNOS, or iNOS) and downstream biochemical effects. It is possible that usual doses of inhaled NO might be neurotoxic for the neonatal brain during both brief and extended periods of time, especially under conditions of oxidative stress and hypoxia–ischemia–reperfusion. In the second part of this review article, we will focus on the known effects of iNO under pathological conditions, with an overall goal to identify areas for future investigation and research.

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AUTHOR CONTRIBUTIONS

D.A. contributed to the concept of the paper, wrote the initial and revised drafts of this manuscript, and approved the final manuscript as submitted; R.S. contributed to the conceptualization of the paper, reviewed and revised the manuscript, and approved the final manuscript as submitted; L.C. contributed to the

conceptualization of the paper, reviewed and revised the manuscript, and approved the final manuscript as submitted.

ADDITIONAL INFORMATION

Competing interests: R.S. is on the Scientific Advisory Council of Mallinckrodt Pharmaceuticals and had no role in the development of this review. D.A. and L.C. have no conflicts of interest to disclose.

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