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FORUM REVIEW ARTICLE

Nitroxyl in the Central Nervous System

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Abstract

Nitroxyl (HNO) is the one-electron-reduced and protonated congener of nitric oxide (NO). Compared to NO, it is far more reactive with thiol groups either in proteins or in small antioxidant molecules either converting those into sulfinamides or inducing disulfide bond formation. HNO might mediate cytoprotective changes of protein function through thiol modifications. However, HNO is a strong oxidant that *in vitro* reacts with glutathione to form glutathione disulfide and glutathione sulfinamide. The resulting oxidative stress might aggravate tissue damage in inflammatory diseases. In this review, we will summarize the current knowledge of how exogenous HNO affects the central nervous system, especially nerve cells and glia in health and disease. Unlike most other organs, the brain is separated from the circulation by the blood–brain barrier, which limits access of many pharmacological compounds. Given that, we will review what is known about the ability of currently used HNO donors to cross the blood–brain barrier. Moreover, considering that the physiology and composition of the brain has unique properties, for example, expression of brain-specific enzymes like neuronal NO synthase, its high iron content, and increased energy metabolism, we will discuss possible sources of endogenous HNO in the brain. *Antioxid. Redox Signal*. 14, 1699–1711.

Introduction

Nitroxyl (HNO) is the one-electron-reduced and protonated congener of nitric oxide (NO). Although initially believed to be a strong acid, laser flash photolysis experiments have shown that NO^- in its triplet and HNO in its singlet form have a much lower acidity (pKa 11.4) (6). Therefore, HNO rather than nitroxyl anion (NO^-) predominates at physiological pH. Although biochemists and physiologists have extensively studied the properties of NO, the physiologic generation and function of HNO still remains enigmatic (23).

As long established for NO, HNO shows vasorelaxant properties and, in addition, has been demonstrated repeatedly to be cardioprotective in experimental animal models (19, 33, 51, 53). Thus, HNO donors might be promising future therapeutics for cardiovascular diseases (33, 51, 53). Although most research regarding the possible endogenous generation of HNO and the action of HNO donors has been focused on the cardiovascular system, little is known about the potential sources of HNO in the central nervous system (CNS). Due to its unique gene expression pattern, some pathways of HNO generation might be specific to the CNS. Moreover, the prerequisites for and consequences of pharmacological HNO delivery to the CNS might differ from those of other tissues.

One of the prominent properties of nerve cells is their electrical excitability. In contrast to excitable myocytes, neuronal electrical excitability is involved in pathways leading to cytotoxicity, that is, excitotoxicity, which is neuron specific. Moreover, the electrical excitability of nerve cells is the basis for the high energy demand of the brain, because it almost completely depends on oxidative phosphorylation to generate energy, implying a high production of reactive oxygen species by mitochondria. This and the fact that the brain contains a high concentration of polyunsaturated fatty acids, which are highly susceptible to peroxidation, and has relatively high amounts of iron and copper, which catalyze the generation of certain reactive oxygen species, render the brain very sensitive to oxidative stress (9). HNO is much more reactive than NO and acts as a pro-oxidant (53). Thus, HNO might act differently in the brain compared to other organs. Moreover, for drug delivery to the brain, certain physicochemical requirements of HNO donors have to be considered as the brain is separated from the circulation by the blood–brain barrier (BBB).

In this review, we will briefly summarize the unique properties of HNO and discuss the proposed pathways of endogenous HNO production with respect to its potential presence in the CNS. Moreover, we will give an overview of which HNO donors have been used to study the effect of

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HNO on brain function *in vivo* and nerve and glia cells *in vitro* to date, including their potential ability to cross the BBB and their mode of release at physiological conditions. We conclude that evidence is emerging that at least rapidly releasing HNO donors are prone to induce neurotoxicity.

Chemistry and Biology of HNO

NO and HNO share common characteristics; for example, their vasorelaxant effects are mediated predominantly *via* activation of soluble guanylate cyclase (sGC) and the subsequent generation of cGMP (44). In contrast to NO, which does not show direct reactivity, HNO has been shown to be very reactive with many biomolecules. Its high reactivity makes it impossible to study its chemistry and biological actions directly. Rather, the generation of HNO can be inferred *via* the use of methods that detect specific reaction products of HNO with biomolecules. Thus, using high-performance liquid chromatography (HPLC) with conventional diode array detector to study the reaction of HNO with reduced glutathione (GSH) in cell free systems *in vitro*, it was possible to demonstrate the formation of sulfinamide [$GS(O)NH_2$] (18). Since this compound is specifically generated by the reaction of these two substrates, $GS(O)NH_2$ has been proposed as a valuable biomarker to evaluate the production of HNO in biological systems. Further, a combination of the HPLC method with mass spectrometric analysis has significantly improved the detection of this HNO reaction product. Incubating HNO with labeled GSH [(glycine 1,2-$^{13}C2$, ^{15}N) GSH] elicits strongly enhanced spectra of the corresponding labeled $GS(O)NH_2$ using electrospray ionization–tandem mass spectrometry (79). Therefore, this method provides a more sensitive and specific approach compared to HPLC alone (Fig. 1). Still, the detection of HNO reaction products in cells and *in vivo* remains a great challenge.

Possible Pathways of Endogenous HNO Generation in the Brain

Despite technological developments, endogenous production of HNO *in vivo* and in cultured cells *in vitro* remains to be demonstrated. However, several potential biosynthetic mechanisms have been postulated. Most importantly, it was suggested that HNO could directly be formed as an intermediate in the catalytic turnover of L-arginine by NO synthase (NOS). It could be demonstrated that the synthesis of NO and citrulline from L-arginine *via* neuronal NOS (nNOS) requires the presence of tetrahydrobiopterin (THB). When THB is absent, nNOS catalyzes arginine oxidation to citrulline with consecutive liberation of HNO, not NO (2). Moreover, once HNO is formed from NOS, the enzyme superoxide dismutase (SOD) has been demonstrated to accelerate the conversion of HNO to NO and therefore functions as an important cofactor (29, 60). nNOS is the predominant NOS isoform in neurons and is activated by increased intracellular calcium. nNOS is activated during and propagates the induction of excitotoxicity, a form of neuronal cell death following the overactivation of ionotropic glutamate receptors, which is believed to be very important in brain ischemia (46). Indeed, it could be demonstrated that inhibition or deficiency of nNOS reduces ischemic CNS injury in animal models of ischemic stroke (31). Theoretically, generation of HNO rather than NO might be, at least in part, responsible for NOS-induced tissue damage, if at least one of the two cofactors, THB or SOD, becomes limited for NO generation by NOS in pathological states of the brain. THB depletion in an animal model of fetal cerebral hypoxia was reported to correspond with a higher incidence of severe motor deficits and perinatal death (75). Moreover, Fabian *et al.* reported that THB administration can induce NO release during neonatal cerebral hypoxia-ischemia, suggesting a relative THB deficiency in cerebral ischemia (21). Thus, THB deficiency could theoretically lead to increased HNO production by NOS during cerebral ischemia.

Cerebral SOD levels decrease after focal and global transient cerebral ischemia in animal models (32). While overexpression of the superoxide scavenging enzyme SOD attenuates (37), a reduction of SOD activity exacerbates (34) neuronal cell injury after transient focal and global cerebral ischemia, indicating that SOD protects against ischemic neuronal death. Whether these changes in SOD expression are

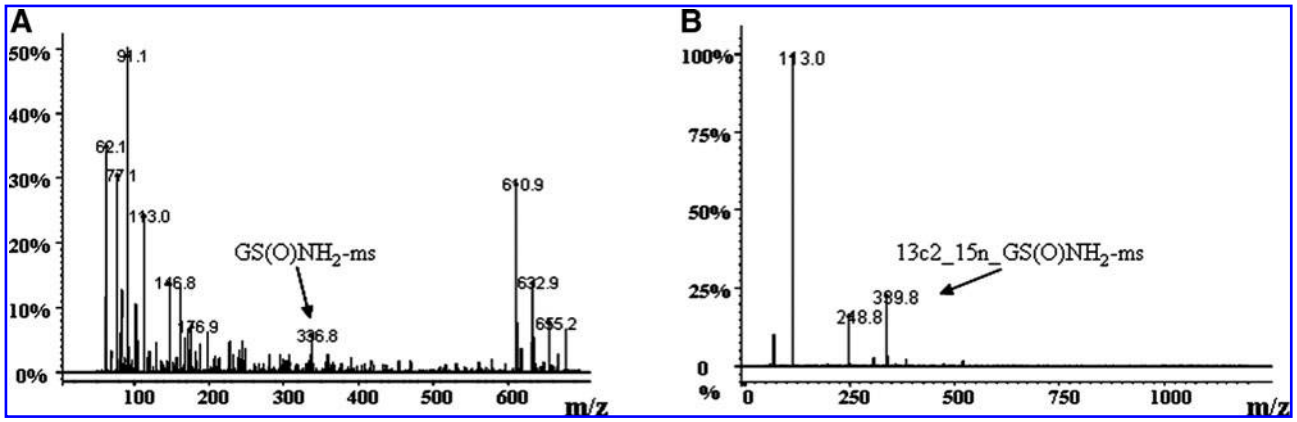

FIG. 1. Detection of reaction products of nitroxyl (HNO) with reduced glutathione (GSH) *in vitro*. Identification of unlabeled **(A)** and labeled **(B)** glutathione sulfinamide ($GS(O)NH_2$) by electrospray ionization–tandem mass spectrometry (ESI-MS) analysis. Incubation of HNO with unlabeled and labeled GSH [containing isotopically labeled glycine ($^{13}C_2$, ^{15}N)] shows significantly enhanced spectra for labeled $GS(O)NH_2$ **(B)** compared with unlabeled $GS(O)NH_2$ **(A)** using ESI-MS (unpublished data).

associated with altered NO liberation *via* NOS has not been reported. However, we conclude that as a strong activation of NOS, a relative THB deficiency and a decrease in SOD activity have been reported in cerebral ischemia, HNO might possibly be produced under these conditions (Fig. 2).

Another potential source of HNO in the brain is hydroxylamine (NH_2OH). NH_2OH can be viewed as a two-electron-reduced product of HNO and a three-electron-reduced product of NO. We recently showed that NH_2OH oxidation by a variety of heme proteins results in HNO formation (16) (Fig. 3). The most efficient release of HNO was observed with the heme protein myeloperoxidase (MPO) (16). Of note, MPO is a myeloid-specific enzyme that is normally absent in the healthy brain, where the microglia, brain cells of myeloid origin, are quiescent. However, strong expression of MPO has been demonstrated in activated microglial cells during intracerebral inflammatory responses, for example, surrounding senile plaques in Alzheimer's disease (58) or in multiple sclerosis (26). Expression of MPO in microglia is thought to exacerbate the adverse effect of neuroinflammation (39). Thus, in areas of inflammatory activation in the brain, MPO, a potential catalyzer of NH_2OH-derived HNO formation, is present.

What are the potential sources of the HNO precursor NH_2OH required for MPO-mediated HNO formation in the brain? One reaction that liberates NH_2OH is the reduction of S-nitroso-GSH (GSNO) to GSH and NH_2OH (8). This reaction is catalyzed by specific enzymes, GSNO reductases, including carbonyl reductase 1 and GSH-dependent formaldehyde dehydrogenase/class III alcohol dehydrogenase (8), both of which are expressed in the brain (45). The required precursor, GSNO, is a product of NOS-mediated S-nitrosylation of the cysteine residue of the ubiquitous small molecule antioxidant GSH. Similarly, cysteine residues of proteins can be S-nitrosylated and GSNO can be produced by transnitrosylation of GSH and proteins (30). Of note, activated microglia express high levels of inducible NOS (iNOS) and in areas of intense microglia activation, abundant protein nitrosylation has been documented (5). Thus, it seems possible that iNOS-induced nitrosative stress during inflammation in the CNS generates GSNO. Subsequently, NH_2OH might be generated by GSNO reductase, which in turn can yield HNO in activated inflammatory cells in the brain where MPO is expressed (Fig. 4).

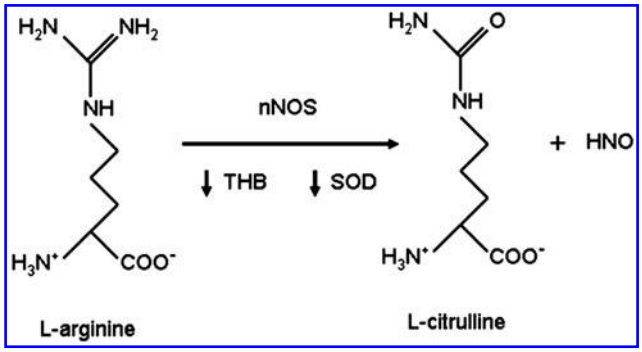

FIG. 2. Putative endogenous HNO formation by neuronal nitric oxide synthase (nNOS). Downregulation of the cofactors tetrahydrobiopterin (THB) or superoxide dismutase (SOD) leads to the conversion of L-arginine to L-citrulline and HNO by nNOS.

However, there is a potential alternative pathway by which an S-nitrosylated thiol can release HNO directly without NH_2OH as an intermediate. This pathway requires an S-nitrosylated thiol with an adjacent intramolecular thiol group (4). Therefore, it is not surprising that two dithiols ubiquitously present in cells, thioredoxin and dihydro lipoic acid, can denitrosylate GSNO and S-nitrosylated proteins *via* a transnitrosylation reaction with one of their cysteine residues. In a second step, HNO is released in parallel with intramolecular disulfide formation of the two adjacent cysteine residues (66) (Fig. 4).

Another potential source of HNO in nerve cells is cytochrome c, a heme protein loosely associated with the inner membrane in mitochondria and involved in oxidative phosphorylation. Purified cytochrome c can reduce NO to NO^-, leading to the formation of HNO at physiological pH (63). Neurons are rich in mitochondria due to their high energy demand. One of the hallmarks of ischemia-induced neuronal death and neuronal apoptosis in neurodegenerative diseases is the release of cytochrome c by the mitochondria (49). As excitotoxicity is involved in cerebral ischemia and neurodegeneration (13), the subsequent calcium-dependent activation of nNOS (see above) will lead to a pathophysiologically relevant generation of NO during these insults. It is conceivable that the presence of cytosolic cytochrome c and high levels of NO might lead to the generation HNO under these circumstances.

In summary, several pathways of endogenous HNO production can be envisioned in the brain. Induction of excitoxicity, for example, during cerebral ischemia, and neuroinflammation are the most likely pathophysiological states where high levels of HNO production seem to be possible.

Properties of Different HNO Donors Used to Study the Effects of HNO on Nerve Cells *In Vitro* and the Brain *In Vivo*

The high reactive potential and potentially rapid autodimerization of HNO necessitate the utilization of HNO donors to study its action *in vitro* and *in vivo*. Different chemical classes of HNO-generating and -releasing compounds have been described. Here, we will discuss only HNO donors that have been employed in investigations of neurons and glia *in vitro* and brain physiology and pathophysiology *in vivo* (Table 1).

More than a century ago, the Italian chemist Angelo Angeli described the release of HNO by an oxide diazeniumdiolate, which was thereafter named Angeli's salt (AS) (56). Currently, AS or sodium trioxodinitrate ($Na_2N_2O_3$) is the most utilized and well-characterized HNO donor. Most studies investigating the role of HNO in neuronal and brain physiology are using AS (Table 1). Although AS is quite stable in alkaline and cold conditions, it spontaneously generates HNO at physiological pH and temperature (56). In the range between pH 4 and 8, the decomposition of AS is independent of pH. Importantly, the reaction is assumed to have a half-life of 2.5 min at physiological conditions, thereby releasing almost instantly micromolar concentrations of HNO (Table 1). The decomposition of HNO releases an equivalent amount of nitrite, which could contribute to hypoxic signaling, vasodilatation, and cytoprotection (25). Therefore, decomposed AS has been

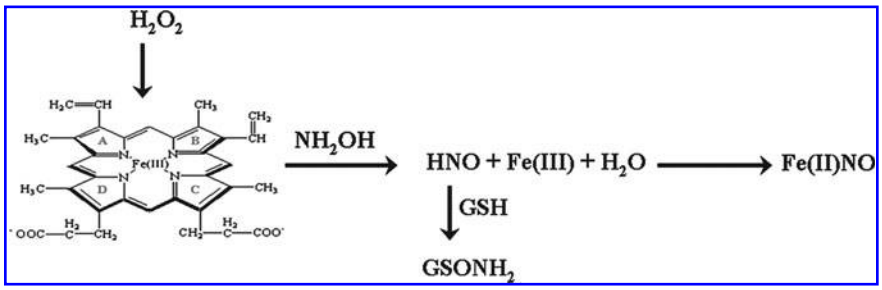

FIG. 3. HNO formation from peroxidation of hydroxylamine (NH_2OH) by heme proteins. In the presence of hydrogen peroxide (H_2O_2), a variety of heme proteins are able to induce the oxidation of NH_2OH to HNO. Once formed, HNO can either react with the ferric heme [Fe(III)] to produce a ferrous nitrosyl complex [Fe(II)NO] or escape from the protein pocket and react with GSH to form sulfinamide ($GS(O)NH_2$), which indicates the amount of free HNO that can be trapped by GSH.

commonly used as a control to account for confounding effects of its by-products.

Although N-hydoxysulfenamide (Piloty's acid) has been utilized to study HNO function in cell paradigms of neuronal excitotoxicity (35), the importance of Piloty's acid is limited by its chemical properties (53). In contrast to AS, Piloty's acid releases HNO in a pH-dependent manner only generating significant amounts of HNO at a pH level higher than 12. Further, under physiological conditions, the generation of NO exceeds by far the production of HNO. Therefore, Piloty's acid can be considered a strong NO donor and weak HNO donor under physiological conditions (78).

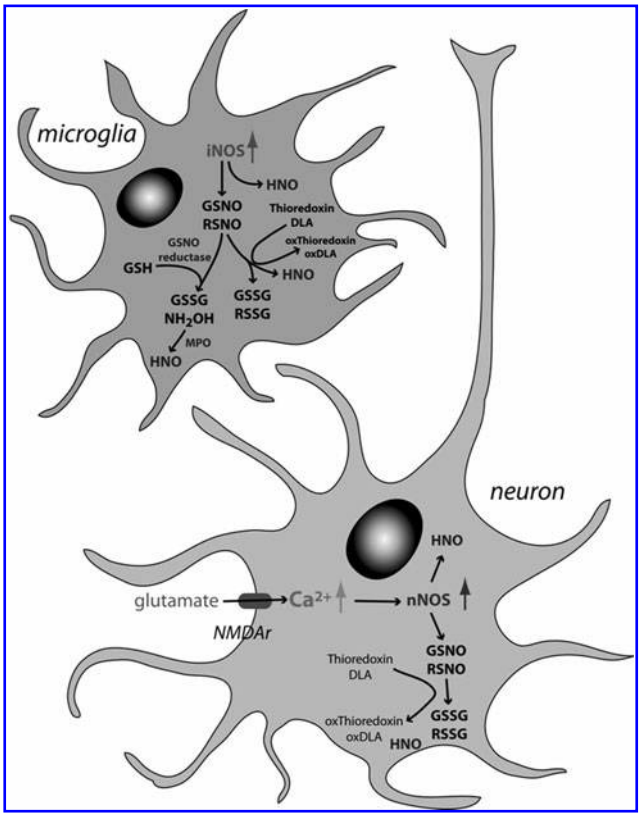

FIG. 4. Potential mechanisms of HNO generation in brain. In neurons, calcium influx (Ca^{2+}) by activation of N-methyl-D-aspartate receptors (NMDAr) activates nNOS, which might either directly produce HNO or leads to S-nitrosylation of GSH to S-nitroso-GSH (GSNO) or S-nitrosylations of other protein and nonprotein thiols (RSNO). S-nitrosothiols can be converted to HNO by dithiols like thioredoxin and dihydro lipoic acid (DLA), leading to their oxidation (oxThioredoxin, oxDLA). In activated microglia, another potential pathway of HNO generation is the direct production of HNO by inducible NOS (iNOS), the reduction of GSNO by GSNO reductases, which yields NH_2OH and GSH disulfide (oxidized glutathione [GSSG]). NH_2OH can be converted into HNO by myeloperoxidase (MPO).

Another supplier of HNO is cyanamide, which has gained much attention in the treatment of alcoholism (53). The chemical modification and inhibition of aldehyde dehydrogenase, a key enzyme in alcohol metabolism, decreases the tolerance to alcohol functioning as an aversion treatment. After oxidative bioactivation of cyanamide by the ubiquitiously expressed enzyme catalase, the intermediate N-hydroxycyanamide is generated. This unstable compound spontaneously degrades to yield HNO and cyanide. The equimolar generation of HNO and its biologically active by-product cyanide has limited its use.

Acyl nitroso compounds react with nucleophiles to yield HNO, but these highly reactive intermediates must be generated *in situ* (36). Recently, Sha *et al.* reported evidence that the acyl nitroso compound, 1-nitrosocyclohexyl acetate, hydrolyzes to produce cyclohexanone, HNO, and acetate (62). The spontaneous decomposition showed strong pH dependence with a half-life of $\sim$8 min in 0.025 *M* NaOH but a relative stability at pH 7.4. It was shown that the modification of the acyl group regulates the stability of the acyl nitroso compounds without affecting their ability to act as HNO donors. The high stability at physiological pH might provide a sustained release of very low amounts of HNO. Assuming that HNO is indeed generated endogenously, a sustained release HNO donor might mimic the natural action of endogenous HNO more closely than AS. Thus, acyl nitroso compounds are highly promising candidates for further exploration of HNO effects on nerve cells *in vitro* and the CNS *in vivo*. However, these studies must be controlled rigidly for effects of their by-products.

The CNS is separated from the circulation by the BBB. Moderately lipophilic drugs cross the BBB by passive diffusion, but the hydrogen binding properties of drugs can significantly influence their CNS uptake profiles (52). Polar or charged molecules are generally poor CNS agents unless they undergo active transport across the BBB. It has been estimated that more than 98% of all small molecule drugs do not cross the BBB (57). Therefore, the bioavailability of systemically administered HNO donors to the CNS can be very different compared with other organs.

Table 1. Nitroxyl Donors Utilized in Investigations of Neuronal and Brain Physiology *In Vitro* and *In Vivo*

HNO donor	*By-products*	In vitro *studies*	In vivo *studies*	*Half-life at pH 7*	*tPSA (Å^2)*	*CLogP*
Angeli's salt ($Na_2N_2O_3$)	Nitrite anion (NO_2^-)	Neuronal cell cultures (12, 35) Mixed glial and neuronal cultures (28, 72)	Cerebral ischemia and reperfusion injury in mouse (12) Intranigral infusion in rat (72)	2.5 min (33, 53)	93.22	−1.332
Piloty's acid	Nitric oxide (NO)	Neuronal cell cultures (35)		>3 h (78)	66.4	−0.259
Cyanamide (CH_2N_2)	Cyanide(CN^-)		Cerebral lipid peroxidation in mouse (27)	Not available	49.81	−1.316

CLogP, calculated octanol/water partition coefficient; HNO, nitroxyl; tPSA, total polar surface area.

However, pharmacokinetic data concerning the CNS bioavailability of HNO donors have not been published. BBB penetration is optimal when the octanol/water partition coefficient (LogP), a measure for lipophilicity, is between 1.5 and 2.7 (52). The calculated LogP (CLogP) of the commonly used HNO donors, AS, Piloty's acid, and cyanamide, are all in the negative range (Table 1), indicating poor BBB permeability. A measure for hydrogen bonding capacity and polarity is the total polar surface area (tPSA), which should be less than 60–70 Å^2 for efficient CNS drugs (52). The tPSA of AS is considerably larger (Table 1). Of note, acyl nitroso compounds like 1-nitrosocyclohexyl acetate (62), although their molecular mass is much higher, might have favorable properties since the hexane ring provides some lipophilicity. Indeed the CLogP for 1-nitrosocyclohexyl acetate is 1.482 and the tPSA is 55.73 Å^2.

Importantly even therapeutics, which have limited access across the BBB, will reach therapeutically relevant intracerebral concentrations if applied in neurological diseases like stroke, multiple sclerosis, Alzheimer's disease, and CNS infections, which all are associated with deteriorated BBB function (76). Numerous studies have shown *in vivo* and *in situ* that BBB opening occurs after cerebral ischemia (1). Although the disruption of the BBB was initially believed to be an acute, unselective, and unspecific phenomenon, recent studies indicate that initially the break down of BBB function is restricted to small molecules such as albumin within the first 6 h after cerebral ischemia and extends to larger molecules such as dextrans and eventually even red blood cells within 24 h (48). Therefore, the relatively small, although rather hydrophilic, HNO donors are expected to readily cross the BBB early in cerebral ischemia. The BBB disruption in multiple sclerosis, an inflammatory demyelinating disease of the CNS, represents a major histological and neuroradiological hallmark (43). In contrast, the increased BBB permeability in Alzheimer's disease is much less understood (43, 76). In summary, it is anticipated that HNO donors will enter the diseased brain more readily than the healthy brain.

In addition, systemic administration of pharmacological compounds might influence the BBB permeability. This was demonstrated for different substances known to release various NO redox forms (NO, NO^-, and NO^+) in studies of perfused rodent brains *in situ* (10). Among seven tested drugs, AS had the second strongest effect on BBB permeability, eliciting an 18% disruption when normalized to hyperosmotic mannitol. In comparison to AS, no considerable BBB disruption could be observed with Piloty's acid (10). Thus, HNO donors themselves might increase BBB permeability. For therapeutic use, this effect could theoretically be desirable if the ability of HNO donors to increase BBB permeability will facilitate their entry into the brain and thereby confer protection. However, this effect is a major disadvantage when effects specific for intracerebral HNO generation are studied as increased BBB will lead to increased influx of multiple small (*e.g.,* urea, creatine, and glucose) and large molecules (*e.g.,* albumin), each of which might potentially affect brain physiology.

Exogenous HNO Applied by HNO Donors *In Vitro* and *In Vivo*

The effect of exogenous HNO on cellular function of neurons and glial cells in vitro

HNO has been shown to be remarkably reactive toward most free thiols and thiol proteins (16, 17, 35, 41, 77). Considering the high concentration of free thiols in cells (*e.g.,* GSH, 1–10 m*M*) (59), and the high reactivity of HNO with thiols (2×10^6 M^{-1} s^{-1}), HNO might mainly react with GSH. However, several reports have shown that selected thiol proteins can be substantially modulated at very low HNO levels without altering free thiol concentrations (41), indicating that HNO preferentially reacts with thiol groups of specific proteins some of which are enzymes and neurotransmitter receptors (Fig. 5). Thus, many of the observed actions of HNO might result from the reaction with specific protein thiols or by altering the cellular redox state by reacting with GSH.

Indeed in the cardiovascular system, many of the actions of HNO such as its positive inotropic, calcium mobilizing, vasorelaxant, and cardioprotective effects have been associated with HNO-dependent oxidation of thiol-containing residues (33, 38, 69). In contrast, the effect of HNO on neuronal cells and brain physiology still remains ill defined (53, 68).

However, evidence exists that HNO may also target thiol groups in the CNS to modulate neuronal function. Thus, glutamate is the major excitatory neurotransmitter in the CNS. Glutamate activates different classes of ionotropic glutamate receptors of the kainate, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) subtype as well as metabotropic glutamate receptors. Excitotoxicity is a form of neuronal cell death evoked by overactivation of glutamate receptors, mainly of the NMDA type, with subsequent calcium overload and is thought to be an important mechanism of neuronal cell death in many CNS diseases (13). Early *in vitro* experiments suggested that HNO modifies thiol residues in the NMDA receptor, mainly Cys-399

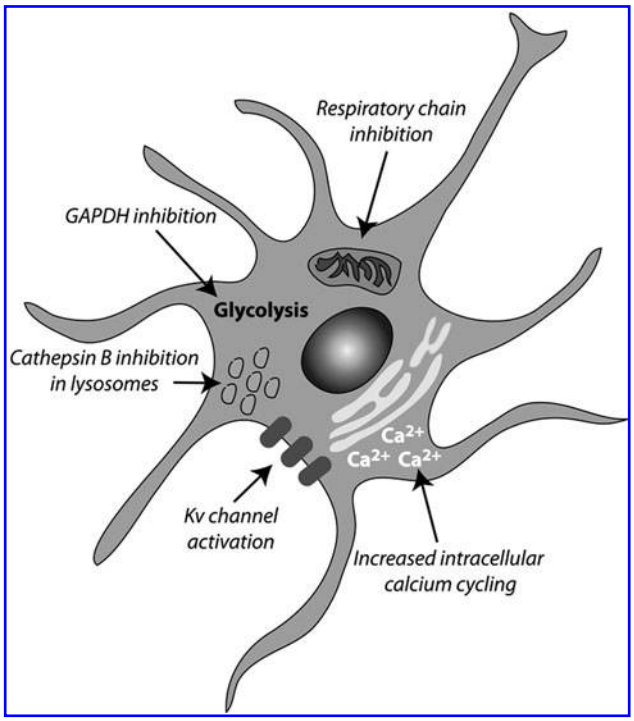

FIG. 5. Putative metabolic enzymes and ion channels targeted by HNO in neuronal physiology and pathophysiology. Kv channel, voltage-gated potassium channel; GADPH, glyceraldehyde-3-phosphate dehydrogenase; Ca^{2+}, calcium ion.

in the NR2A subunit, as the effect was substantially decreased when this amino acid was mutated (35). It was shown that the HNO-induced modifications attenuate NMDA-induced activation, calcium influx, and finally excitotoxic neuronal death (35). However, these findings by Kim *et al.* should be interpreted with caution given that the HNO-releasing drugs utilized and the cell death paradigm used to investigate neuroprotection were suboptimal. All three utilized drugs, Oxi-NO, Sulfi-NO, and Piloty's acid, are very inefficient HNO donors at physiological pH, with millimolar concentrations of these donors required to release low micromolar levels of HNO (24). Further, a major limitation of the study was the use of NMDA to induce excitotoxicity in neuronal cultures. Unlike the neurotransmitter glutamate, NMDA is neither taken up and thereby inactivated by excitatory amino acid transporters (EAATs), nor does it activate AMPA, kainite, and metabotropic glutamate receptors. Of note, we saw no protective effect of the HNO donor AS against excitotoxicity induced with glutamate in pure cortical neurons (12). Moreover, in HEK293 cells transiently transfected with NMDA receptors of different subunit compositions, Colton *et al.* reported distinctly different effects of HNO on glutamate-activated NMDA receptor currents compared to the previous results (15). Cells expressing NMDA receptors composed of NR1-NR2A subunits show a dramatic, but reversible increase in glutamate-induced steady state whole cell currents upon perfusion with AS. In contrast, NR1-NR2B-containing NMDA receptors were only moderately inhibited by AS (15).

Other groups have shown that direct exposure of neuronal cell cultures to the HNO donor AS alone resulted in concentration-dependent cell death (28, 72). In primary mixed cortical glial/neuronal cell cultures, millimolar concentrations of AS were required to elicit significant cell death with neurons being much more sensitive than astrocytes (28). In contrast, low micromolar concentrations were already sufficient to decrease viability in pure cultures of cortical neurons (12, 72). Although studies directly comparing cortical neuronal cultures with and without glial cells have not been published, these data suggest that astrocytes play a protective role in HNO-induced neurotoxicity. However, death of dopaminergic neurons, assayed by a loss of dopamine uptake, has been observed at AS concentrations of 40–200 μM in primary mesencephalic neuronal cultures grown in the presence or absence of glial cells (72). Irrespective of the neuronal subtypes and the role of astrocytes, various potential mechanisms, such as excitotoxicity and oxidative stress, might contribute to the neurotoxic effect of AS (Fig. 6). The detrimental consequences of AS exposure were partially reversed by blocking both NMDA and AMPA/kainate receptors with MK-801 and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), respectively, with an additive effect when both inhibitors were combined (28). Further, inhibition of exocytosis by tetanus toxin partially ameliorated the toxic effect of AS on mixed neuronal and glial cultures (28). Taken together, it seems that high concentrations of the HNO donor AS induce a neuron-specific toxicity that is partially mediated by synaptically released glutamate and its subsequent activation of diverse ionotropic glutamate receptors. Whether AS induces excessive release of glutamate or impedes its uptake into astrocytes has not been demonstrated. Astrocytes take up synaptically released glutamate *via* EAATs. Of note, EAATs have been demonstrated to be inhibited by oxidants through intramolecular disulfide bond formation (70). Whether HNO increases extracellular glutamate by inhibiting EAATs or by augmenting vesicular excitatory neurotransmitter release remains to be explored. The hypothesis that impaired glutamate uptake by astrocytes contributes to HNO toxicity would be consistent with the findings that astrocytes do not modulate HNO toxicity in nonglutamatergic neurons (72).

In addition to its toxic effect on cultured neurons, HNO derived from 300 μM AS exacerbated cell death in a mixed cortical culture exposed to oxygen–glucose deprivation (OGD), an *in vitro* paradigm for ischemic stroke (12), which in part involves excitotoxic mechanisms. Moreover, in immature cortical neurons still lacking functional ionotropic glutamate receptors, we showed that HNO exacerbates oxidative glutamate toxicity, a neuronal cell death paradigm distinct from excitotoxicity (3), at concentrations as low as 50μM AS (12). This effect was HNO-specific and independent of AS byproducts like nitrite, as decomposed AS had no effect on oxidative glutamate toxicity. In oxidative glutamate toxicity, excess extracellular glutamate inhibits uptake of the rate-limiting amino acid for GSH synthesis, cystine, *via* the glutamate/cystine antiporter system x_c^-. Thereby, glutamate induces GSH depletion, subsequent oxidative stress, and finally cell death (3).

GSH is the most important antioxidant in the brain and the oxidized glutathione (GSSG)/GSH ratio is the major determinant of the cellular redox state (59). To ameliorate oxidative stress, excess GSSG is rapidly exported from brain cells (18). HNO has pro-oxidant properties and in the presence of excess GSH it leads to the formation of GSH disulfide (GSSG) (17). In

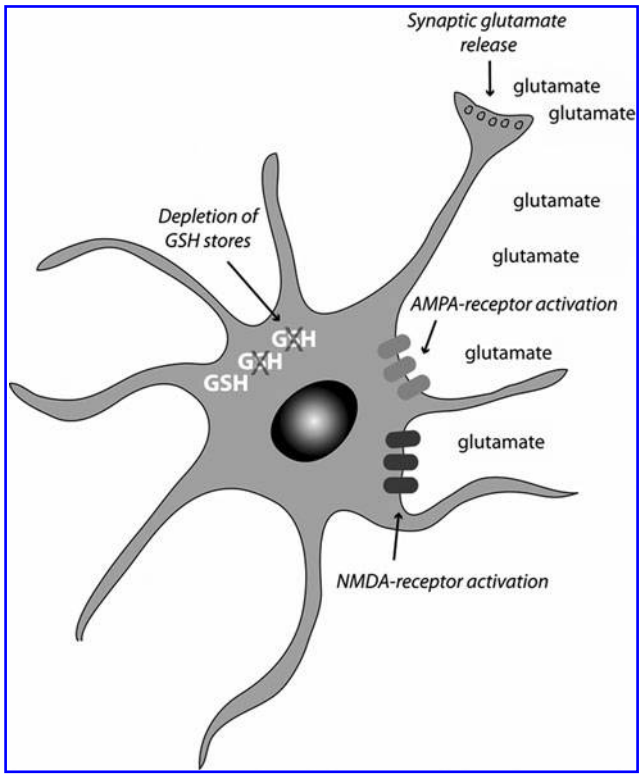

FIG. 6. Pathways by which HNO can induce or aggravate neurotoxicity. HNO-induced synaptical release of glutamate can activate NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and thereby induce or exacerbate excitotoxicity. Moreover, the pro-oxidant properties of HNO can directly deplete cellular pools of GSH and thereby impair the antioxidative defense.

Chinese hamster V79 fibroblasts, the toxicity of AS was enhanced prominently when GSH levels were reduced by inhibition of GSH synthesis (77), confirming a relationship of intracellular GSH and HNO toxicity. In brain homogenate, GSH reduced AS-induced generation of hydroxyl adducts, indicating a protective role of GSH against the pro-oxidant properties of HNO (72). In summary, GSH levels seem to be an important influence on the cytotoxic effect of HNO in nerve cells and HNO might oxidize cellular GSH to GSSG.

Hypothetical additional targets of HNO in nerve cells

The brain is the organ with the highest relative energy consumption and almost completely relies on oxidative phosphorylation for its energy demands. Thus, inhibition of energy metabolism is a very critical aspect when possible effects of HNO on brain physiology are discussed. Mitochondrial dysfunction has been attributed a major role in oxidative stress and mechanisms of neuronal cell death (49). On one hand, mitochondria are capable of converting HNO into NO and NO into HNO (63). On the other hand, HNO hampers oxidative phosphorylation through the inhibition of the mitochondrial complexes I and II (65). The concentration-dependent reduction of the respiratory chain likely involves modifications of specific thiol residues in mitochondrial proteins. Once again, GSH might play a central role in the pathophysiology of HNO. While the treatment of mitochondria with HNO results in the loss of GSH equivalents, the inhibition of complex II activity was completely reversed by adding high concentrations of GSH (65). AS did not directly inhibit complex IV (cytochrome c oxidase) activity in this study using isolated liver mitochondria (65). In contrast, an HNO-generating mixture of ferrocyanide and NO demonstrated a selective inhibition of cytochrome c oxidase in rat brain mitochondria (50). Impairment of oxidative phosphorylation would leave neurons dependent on glycolysis as their sole energy source to generate energy from glucose, but in addition to inhibited oxidative phosphorylation, HNO donors could also impair glycolysis. In yeast, *Saccharomyces cerevisiae*, HNO irreversibly inhibited the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH)—an effect that was suggested to involve a thiol residue in the catalytic site (41). Intriguingly, HNO-mediated inactivation of GAPDH was observed at HNO donor concentrations that were too low to change the redox state of intracellular GSH, indicating that some protein thiol groups preferentially react with HNO (41). Most enzymatic steps after GADPH activation represent exothermic reactions synthesizing high-energy compounds; therefore, GADPH inhibition would significantly decrease glycolytic production of high-energy molecules. Taken together, some of the neurotoxic effects of HNO might be mediated by an impairment of energy metabolism at the level of glycolysis and mitochondrial oxidative phosphorylation.

In cardiovascular pathologies, HNO has already been extensively studied in experimental animal models of heart failure and ischemia-reperfusion injury (33, 38, 69). The positive ionotropic effect depends on increased intracellular calcium cycling *via* HNO-mediated activation of both the ryanodine receptor (RyR) and sarco/endoplasmic reticulum calcium-ATPase (SERCA) (38, 69). The three subtypes of RyRs (type 1, 2, and 3) are important intracellular calcium release channels, which are located in the membrane of the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER). The RyR2 is predominantly expressed in heart. Different studies have shown that HNO activates RyR2, releases calcium from SR vesicles, and increases calcium transients in isolated murine myocytes (69). In addition to RyR2 activation, AS increases the open probability of the skeletal muscle RyR, RyR1, reconstituted in lipid bilayers (11). In contrast to cardiac and skeletal muscle, the brain expresses all three RyR isoforms. Although experimental evidence is lacking, it seems plausible that activation of RyRs is part of the effect of HNO in the CNS. HNO-induced activation of neuronal RyRs can be expected to perpetuate calcium-induced calcium-release (CICR). Besides increased calcium release *via* activated RyRs, enhanced intracellular calcium cycling in myocytes has been attributed to an augmented calcium re-uptake *via* increased SERCA activity (38, 69). Enhanced calcium transients are the consequence of unaltered calcium content of the ER and amplified CICR (Fig. 7). Whereas calcium is responsible for the positive ionotropic and lusitropic effects of HNO in the heart, calcium itself fulfils distinct functions as a versatile and ubiquitous second messenger in excitable and nonexcitable cells of the CNS, regulating gene transcription, synaptic modulation, and neuronal excitability. Therefore, increased calcium transients would amplify extracellular neurotransmitter concentrations (*e.g.*, glutamate and dopamine), alter membrane potential and elicit action potentials, consequently consuming high

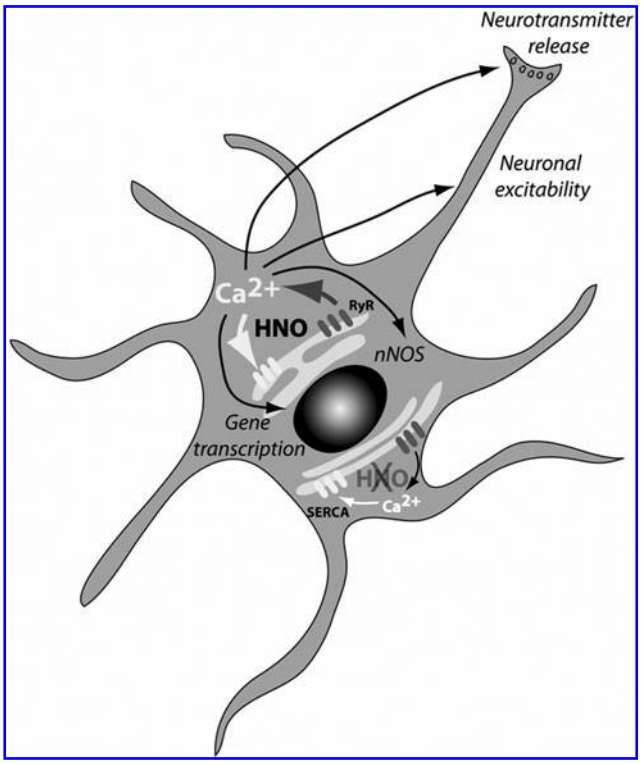

FIG. 7. HNO enhances calcium cycling *via* activation of ryanodine receptor (RyR) and sarco/endoplasmic reticulum calcium-ATPase (SERCA). Amplified intracellular calcium transients might potentially increase neurotransmitter release, neuronal excitability, gene transcription, and energy expenditure.

amounts of energy. Further, calcium as a pleiotropic second messenger is involved in numerous protective but also detrimental processes in nerve cells and glia. In summary, the overall effect of the multifaceted process of HNO-induced calcium release remains difficult to predict.

The vasorelaxant effect of HNO is mediated predominantly by activation of sGC, production of intracellular cGMP, and subsequent modulation of voltage-gated potassium channels (22, 44). Multiple types of voltage-gated potassium channels are expressed in the brain. Opening of voltage-gated potassium channels can hyperpolarize or repolarize cellular membranes (43). Thereby, they counteract the action of sodium and calcium channels and, thus, dampen neuronal excitability. As increased excitation is linked to increased intracellular calcium, activation of voltage-gated potassium channel indirectly decreases intracellular calcium levels. Thus, in theory, an ability of HNO to open neuronal voltage-gated potassium channels may serve to limit neuronal excitability. Using macrophages, it was demonstrated that the lysosomal protease cathepsin B is another target of HNO-mediated inhibition (73, 74). Interestingly, cathepsin B, which is expressed in the CNS, has been shown to be involved in the proteolytic degradation of the amyloid-β peptide and activation of cathepsin B by genetic inactivation of its endogenous inhibitor cystatin C has been shown to decrease amyloid-β plaque burden and improve memory deficits in animal models of Alzheimer's disease (67). Hypothetically, chronic inhibition of cathepsin B by increased endogenous HNO production might favor lysosomal dysfunction and increase the accumulation of amyloid-β plaques.

In summary, multiple HNO targets defined in other cells or tissues are also present in the CNS and could potentially be involved in the action of HNO (Fig. 5).

The effects of exogenous HNO on brain function and vulnerability in vivo

While *in vivo* studies of the effect of HNO on the CNS are scarce, evidence to date suggests that HNO has neurotoxic actions in the setting of Parkinson's disease, amyotrophic lateral sclerosis (ALS), and stroke. Parkinson's disease is a neurodegenerative disease that preferentially involves the dopaminergic neurons of the substantia nigra located in the midbrain. Oxidative stress has been implicated in the pathophysiology of Parkinson's disease (49). Moreover, GSH depletion was repeatedly shown in Parkinsonian midbrains (61). Vaananen *et al.* reported that stereotactic injection of AS (400 nmol) in the substantia nigra resulted in millimolar concentrations at the infusion site and induced an 85% depletion of dopaminergic neurons within 7 days after injection. In contrast, the structurally similar compound sulphononoate or the end product of AS decomposition, sodium nitrite, had no effect on the viability of dopaminergic neurons (72). At a lower dose of 100 nmol, the effect of AS injection was delayed, but progressive with a 15% and 56% loss of dopaminergic neurons after 7 and 50 days, respectively.

Mutation of SOD1 causes a type of hereditary ALS, a fatal degeneration of motor neurons (20). In the ALS spinal cord, increased nitrotyrosine levels are found, indicating increased production of reactive nitrogen species (7). SOD has been implicated in the conversion of HNO into NO (29, 60). The SOD1 mutations in familial ALS can cause an increased reduction capacity of the enzyme's Cu-ion (20) and it has been hypothesized that this might decrease the conversion of HNO to NO and shift the equilibrium between these nitrogen oxides toward HNO (40). This prompted Vaananen *et al.* to test the effect of AS delivered intrathecally on spinal cord motor function and expression of proteins indicative of spinal cord damage (71). A bolus of 10 μmol AS injected into the cerebrospinal fluid compartment surrounding the spinal cord induced delayed but continuous deterioration of motor function, indicating progressive motoneuron dysfunction. In contrast, no relevant sensory disturbances were detected. Thus, the toxicity of AS was motoneuron specific. NO donors and decomposed AS did not induce any effect arguing in favor of a specific HNO effect. Histologically, it was shown that intrathecal HNO release by AS induces increased astrogliosis with increased expression of glial fibrillary acidic protein (GFAP), fibroblast growth factor-2 and γ1-laminin, a pattern typical for spinal injury. Moreover, HNO induced the expression of laminin-1 and γ1-laminin in motoneurons. HNO also induced protein nitrosylation detected by an antibody against nitrotyrosine in motoneurons and astrocytes. In contrast to the *in vitro* studies (28), the effect of HNO on motor function was not inhibited by NMDA receptor inhibition. The authors concluded that acute delivery of HNO to the spinal cord has cytotoxic effects and that motoneurons are more susceptible than sensory neurons, resulting in a phenotype resembling ALS.

We could show that systemic administration by intraperitoneal injection of AS significantly increased infarct size in

middle cerebral artery occlusion (MCAO), an animal model of ischemic stroke (Fig. 8) (12). Although we demonstrated that HNO has similar effects in *in vitro* paradigms of cerebral ischemia, like OGD and oxidative glutamate toxicity, the HNO effects in MCAO likely involve additional mechanisms besides direct neurotoxicity. Parenteral AS administration induced systemic oxidative stress indicated by increased levels of the isoprostanes in serum and urine (12). As originally proposed by Feelisch and coworkers, AS administration may exacerbate myocardial ischemia reperfusion injury through endothelial thiol oxidation and resultant increased neutrophil adhesion in affected zones (42). Systemic HNO administered before MCAO may similarly augment oxidative inflammatory interactions at the level of cerebral vessels, leading to an increased recruitment extravasation of neutrophils and monocytes into ischemic brain parenchyma. An additional leukocytic infiltration would worsen the severity of MCAO and increase lesion size. Moreover, as expected, AS significantly decreased mean arterial blood pressure (12). Given residual CNS perfusion in MCAO is critically decreased by hypotension (14), the effect of HNO on the mean blood pressure after AS administration might also contribute to the observed outcome.

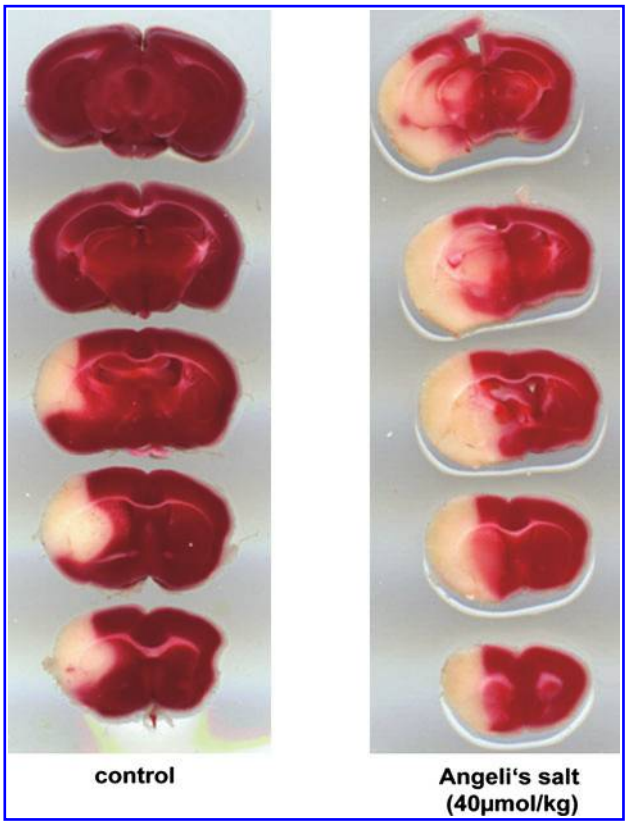

FIG. 8. Systemic administration of the HNO donor Angeli's salt exacerbates ischemic brain injury. Mice were injected intraperitoneally with indicated amounts of Angeli's salt or vehicle (control) directly before induction of transient cerebral ischemia by 1 h of middle cerebral artery occlusion (MCAO). The micrographs show coronal sections of representative mouse brains 2 days after MCAO stained with 2,3,5-triphenyltetrazolium chloride. Red areas mark vital tissue and white areas indicate ischemic brain regions. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Our data show that HNO exacerbates the neurological outcome and the infarct size in a model of cerebral ischemia-reperfusion and HNO might also modulate perfusion and vascular permeability. Entry of HNO or AS into the brain may be facilitated by two mechanisms—the disruption of the BBB after cerebral ischemia and a significant increase of BBB permeability after AS administration (1, 47). Thus, it seems plausible that at least part of the deleterious action of AS can be explained by local action of HNO on brain tissue. This would be consistent with the neurotoxic effects of HNO derived from AS when delivered directly to CNS (71, 72). Thus, we postulate that increased oxidative stress and subsequent depletion of antioxidant reserves aggravates neurotoxicity. Importantly, the micromolar concentrations of HNO, shown to be detrimental in the nervous system, have been shown to be cardioprotective (54, 55, 69). Therefore, the distinct effect of HNO on the nervous system must be taken into account when considering HNO as a therapeutic agent in the cardiovascular system.

In summary, HNO delivered by the rapidly decomposing HNO donor, AS, induced direct neurotoxicity or exacerbated brain damage in three independent studies (see above). However, the kinetics and concentrations of HNO released by AS are very different from a proposed endogenous release of HNO.

Summary and Future Perspectives

The effect of HNO on cardiac function has been studied extensively (38, 54, 69). HNO donors are assumed to be promising future therapeutic tools for the treatment of heart failure (38, 53, 54, 69). The effect of HNO on the function of brain cells and its targets are less well understood. Although HNO can modulate NMDA receptor function, classical HNO donors seem to have deleterious effects on nerve cell viability *in vitro* and *in vivo* (12). Most possibly, this effect is, at least in part, mediated by the pro-oxidant effects of HNO. In addition, other pathways such as the modulation of calcium signaling and the impairment of energy metabolism might theoretically contribute to the observed neurotoxic effects of HNO.

Studying the effect of exogenously applied HNO on the brain *in vivo* is complicated by the fact that the brain is separated from the circulation by the BBB, which might impede effective HNO delivery to the brain. However, the BBB is defective in some important diseases of the brain and some classical HNO donors themselves can disrupt the BBB (see above). Whether the latter leads to artefacts independent of the effect of HNO itself remains to be clarified. All *in vivo* studies investigating neuronal survival and HNO used the short-lived donor AS, which releases HNO within a limited time window of $\sim$10 min. We conclude that HNO donors specifically designed to cross the BBB and allow a sustained release of HNO within the CNS are needed to further analyze the role of HNO in the brain *in vivo*.

Several protein targets of HNO have been seen in the heart, vasculature, and other tissues (see above). Most of these targets are also present in brain cells and might be relevant sites of action for HNO in the CNS, leading to changes in neuronal and glial physiology. Further studies are needed to identify the distinct effects of HNO on neuronal survival.

Whether HNO is generated endogenously *in vivo* remains an open question. Several pathways have been postulated by which endogenous HNO can be generated. These include

HNO production by NOS and S-denitrosylation of GSH or proteins, directly by naturally occurring dithiols, dihydro lipoic acid, and thioredoxin, or indirectly by MPO (see above). With respect to the brain, some special characteristics of its physiology and brain-specific enzymes must be kept in mind when asking the question when and where HNO might be generated in the brain in health and disease. First, neurons express nNOS, which is calcium activated. Calcium overload and excessive nNOS activation occur in neurons in response to NMDA receptor activation by glutamate. Second, MPO, which can catalyze the generation of HNO from NH_2OH, is highly expressed in activated microglia and invading immune cells during neuroinflammation. These cells also express high levels of iNOS, which will be required for HNO generation. Taken together, cerebral ischemia and neuroinflammation might be good starting points to look for endogenous HNO generation and to clarify its role. Again, new HNO donors that enable a sustained release of low concentrations of HNO might be an important step to mimic hypothesized endogenous HNO generation.

Still, the most important current challenge lies in developing reliable detection methods of HNO generation and/or reactivity in cultured cells *in vitro* and biological systems *in vivo*. Currently, the formation of N_2O and NH_2OH is used to identify HNO actions *in vivo*. However, this is only indirect evidence of HNO formation. The formation of sulfinamide with proteins such as GAPDH may provide a unique footprint for the formation and activity of this compound (64). Recently, a novel proteomic approach offers a potentially very useful tool in analyzing the reactive targets of HNO from *ex vivo* samples. The development of analytical methods is critically needed for this potential pharmacological agent to advance.

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Abbreviations Used

ALS = amyotrophic lateral sclerosis
AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AS = Angeli's salt
BBB = blood–brain barrier
CICR = calcium-induced calcium-release
CLogP = calculated octanol/water partition coefficient
CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione
CNS = central nervous system
DLA = dihydro lipoic acid
EAATs = excitatory amino acid transporters
ER = endoplasmic reticulum
ESI-MS = electrospray ionization-tandem mass spectrometry
GADPH = glyceraldehyde-3-phosphate dehydrogenase
GFAP = glial fibrillary acidic protein
GSH = reduced glutathione
GSNO = S-nitroso-glutathione
$GS(O)NH_2$ = glutathione sulfinamide
GSSG = oxidized glutathione
HNO = nitroxyl
HPLC = high-performance liquid chromatography
H_2O_2 = hydrogen peroxide
iNOS = inducible nitric oxide synthase
LogP = octanol/water partition coefficient
MCAO = middle cerebral artery occlusion
MK-801 = dizocilpine maleate
NMDA = N-methyl-D-aspartate
MPO = myeloperoxidase
NH_2OH = hydroxylamine
NMDAr = N-methyl-D-aspartate receptors
nNOS = neuronal nitric oxide synthase
NO = nitric oxide
NOS = nitric oxide synthase
OGD = oxygen–glucose deprivation
RyR = ryanodine receptor
SERCA = sarco/endoplasmic reticulum calcium-ATPase
sGC = soluble guanylate cyclase
SOD = superoxide dismutase
SR = sarcoplasmic reticulum
THB = tetrahydrobiopterin
tPSA = total polar surface area

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