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Nitric oxide synthases: targets for therapeutic strategies in neurological diseases

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Abstract. Glutamate excitotoxicity, oxidative stress, and mitochondrial dysfunctions are common features leading to neuronal death in cerebral ischemia, traumatic brain injury, Parkinson's disease, Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis. Nitric oxide (NO) alone or in cooperation with superoxide anion and peroxynitrite is emerging as a predominant effector of neurodegeneration The use of NO synthase (NOS) inhibitors and mutant mice lacking each NOS isoform have provided evidence for the injurious effects of NO derived from neuronal or inducible isoforms. New neuroprotective strategies have been proposed with selective NOS inhibitors for the neuronal (ARL17477) or the inducible (1400W) isoforms or with compounds combining in one molecule selective nNOS inhibition and antioxidant properties (BN 80933), in experimental ischemia-induced acute neuronal damage. The efficacy of these new strategies is well established in acute neuronal injury but remains to be determined in more chronic neurological diseases.

Key words. Nitric oxide; nitric oxide synthase inhibition; neuroprotection; neurological diseases; free radicals; oxidative damage.

Selective or generalized loss of neurons is responsible for many acute neurological disorders as well as chronic neurodegenerative diseases. The cascade of events which leads to neuronal death, probably comparable for the different neuronal insults, is multifactorial. It involves glutamate excitotoxicity, oxidative stress, and mitochondrial dysfunctions. In the last decade, evidence has accumulated implicating an excessive stimulation of glutamate receptors in triggering neuronal degeneration observed in cerebral ischemia, trauma, Parkinson's disease, Huntington's disease, and Alzheimer's disease. The landmark observation that activation of N-methyl-D-aspartate (NMDA) receptors generates nitric oxide (NO) raised the possibility that NO participates in glutamate neurotoxicity. Since then, substantial data have accumulated suggesting that NO, or more precisely excessive NO, production may play a role in neurological diseases. This review addresses our understanding of NO neurotoxicity and some of the current strategies developed to counteract its harmful effects.

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Mechanisms of NO neurotoxicity

NO acts as a neuromodulator in the central nervous system (CNS) and participates in the regulation of diverse physiological processes including brain development, pain perception, neuronal plasticity, memory, and behavior. The three isoforms of NO synthase (NOS), neuronal (nNOS, type I), endothelial (eNOS, type III), and inducible (iNOS, type II) are found in the brain. nNOS is expressed in highly ramified neurons throughout the brain including cerebellum, cerebral cortex, hippocampus, amygdala, and substantia nigra [1]; eNOS is primarily localized in endothelial cells although it has been detected in a small population of neurons; iNOS is not found in healthy tissues but can be expressed after brain insult in astrocytes, neurons, and endothelial cells. When produced in excessive amount, NO switches from a physiological neuromodulator to a neurotoxic effector. Overproduction of NO may occur from nNOS following persistent stimulation of excitatory amino acid receptors [2] mediating glutamate toxicity and/or from iNOS after its induction by diverse stimuli such as endotoxin or cytokines [3]. The predom-

inant mechanism by which NO promotes neuronal death implicates the reaction of NO with superoxide anion ($O_2^{\bullet -}$) to generate the cytotoxic substance peroxynitrite ($ONOO^-$) [4]. This reaction occurs at an extremely high rate, outcompeting superoxide dismutase (SOD) for the substrate superoxide anion. Peroxynitrite is a highly reactive molecule capable of oxidizing proteins, lipids, and DNA and homolytically decomposing to yield even more potent neurotoxins like the hydroxyl radical. In addition, the glutamate transporter is inhibited by peroxynitrite, reducing its uptake function [5]. Since glutamate is not metabolized by extracellular enzymes but removed by cellular uptake, inhibition of uptake maintains NMDA-receptor activation amplifying the neurotoxic cascade. More directly, NO may impair by nitrosylation the function of essential proteins containing iron-sulfur clusters and thiol residues such as aconitase and complex I and II of the mitochondrial respiratory chain. Neuronal death either by apoptosis and/or necrosis occurs by inhibition of key enzymes of the tricarboxylic acid cycle, the mitochondrial respiratory chain, mitochondrial calcium metabolism, or DNA damage with subsequent activation of the energy-consuming pathway involving poly(ADP-ribose) synthetase (PARS; fig. 1). In this cascade, mitochondria, which are very sensitive to the noxious effects of $NO/ONOO^-$, appear to play a pivotal role in the development of neurodegenerative disorders.

Cerebral ischemia and traumatic brain injury

Ischemic stroke usually refers to an occlusion of a blood vessel that results in decreased blood flow and a lack of oxygen and glucose in the brain leading to cell death.

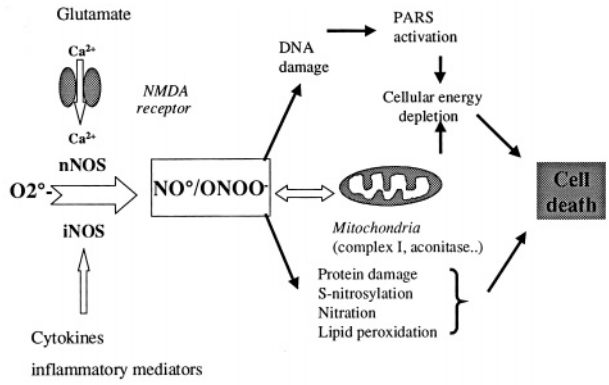

Figure 1. Schematic representation of NO^- and/or peroxynitrite – ($ONOO^-$) mediated neurotoxicity.

Traumatic brain injury results in functional deficits due to both a primary mechanical injury and a secondary ischemic insult that magnifies the initial traumatic damage.

Evidence of NO production

The most detailed evidence that NO plays a role in neurological disorders comes from studies on cerebral ischemia. Increased production of NO in ischemic brain has been observed using a wide variety of techniques based on the detection of NO (NO-sensitive microelectrodes [6], electron paramagnetic resonance, spin trapping [7], nitrites, cGMP assay [8]); NOS proteins (immunocytochemistry, NADPH diaphorase histochemistry [9, 10]), NOS activity (citrulline assay [11]); NOS genes (reverse transcriptase-polymerase chain reaction) [12]. Nitrotyrosine, a specific marker for peroxynitrite-mediated protein nitration [13], which is widely used to assess the involvement of NO and/or superoxide anion in neurological disorders, has been detected during ischemia [14, 15]. In addition, the core region of the infarct, the histopathological site of susceptibility to ischemia, has higher NOS activity than the less vulnerable penumbra [16]. Although less well documented, an increase in NOS activity in a rat model of parasagittal fluid percussion brain injury [17] and the presence of nitrotyrosine in a mouse model of moderate closed head injury [18] have been reported.

Effects of NOS inhibitors

Since the first report of Nowicki et al. [19] in 1991, showing the reduction by N^G-nitro-L-arginine (L-NA) of the cortical infarct volume induced by permanent focal cerebral ischemia in the mouse, the therapeutic potential of NOS inhibitors (fig. 2) has been emphasized. Partial inhibition of NOS activity is sufficient to afford protection in this model of permanent focal cerebral ischemia in the mouse [20] and a protective effect is observed even if treatment is delayed up to 9 h after ischemia in transient focal ischemia in rats [21]. However, conflicting results have been reported on the effect of nonselective NOS inhibitors on ischemia, ranging from reduction to worsening of the ischemic injury. Apart from different experimental paradigms, the discrepancy may be mainly due to the dosage regimens. The few studies applying multiple doses of L-NA showed a bell-shaped curve in global [22], and focal [20, 23] ischemia. This was attributed to the nonselective action of L-NA and to the detrimental inhibition of eNOS by high doses of L-NA. NO generated from eNOS plays a protective role since it is a potent vasodilator and a platelet aggregation and leukocyte adhesion inhibitor and consequently may improve

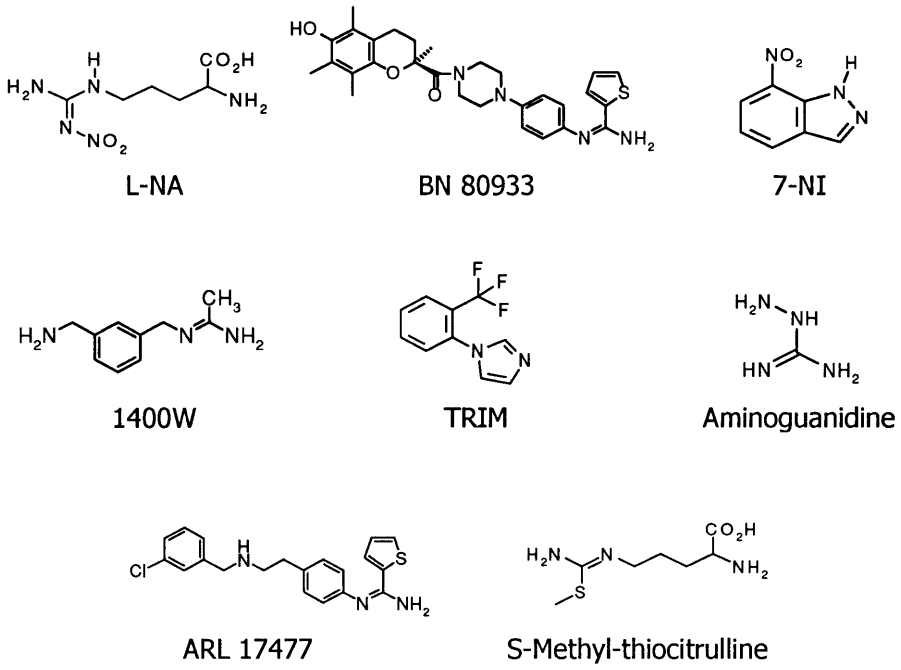

Figure 2. Structure of the nitric oxide synthase inhibitors.

post-ischemic blood flow. Similarly, in traumatic brain injury, nonselective NOS inhibitors afford neuroprotection [24] but may also worsen damage [25] due to prejudicial inhibition of eNOS. This indicated the need for selective NOS inhibitors sparing eNOS activity. Despite an absence of in vitro selectivity in enzymatic or functional [26] assays, 7-nitroindazole was first considered as a paragon of selective nNOS inhibition and was shown to be protective in experimental stroke [27–29] and trauma [24]. Recently, reduced brain ischemic damage has been observed with selective nNOS inhibitors ARL17477 and 1-(2-trifluoromethyl phenyl)imidazole (TRIM) [30, 31]. The observation that the combination of L-NA and an antioxidant (e.g., superoxide anion scavenger) results in a synergistic protection in transient focal ischemia [23], led us to test compounds combining selective nNOS inhibitory activity and antioxidant properties. BN 80933, the prototype of this new series of dual inhibitor, reduced infarct volume and improved behavioral recovery in transient focal ischemia [32].

Inhibitors of iNOS have also been examined as possible neuroprotective agents. Reduced infarct size in experimental ischemia has been shown after treatment with the relatively specific iNOS inhibitors aminoguanidine and agmatine [33] and with 1400W, the most selective iNOS inhibitor presently available [34–36] (Parmentier et al., personal communication). Aminoguanidine was most effective when administered 12 and 24 h after the onset of middle cerebral artery occlusion in rats [37] indicating that expression of iNOS is a post-ischemic event contributing to the delayed extension of the damage.

These observations suggest that both nNOS and iNOS are detrimental in ischemia and indicate the sequential importance of each NOS isoform during evolution of the ischemic process.

NOS-deficient mice

Confirming independently the results obtained with such pharmacological approaches, mice in which the gene encoding nNOS or iNOS was deleted showed reduced damage in response to focal ischemia compared with wild-type mice [38, 39]. In contrast, highlighting the protective role of eNOS, mutant mice deficient in eNOS developed larger lesions after middle cerebral artery occlusion [40].

NO and neurodegenerative diseases

The feature shared by neurodegenerative diseases is slow and gradually evolving neuronal death of selective neuronal populations. These disorders can occur sporadically or, in some instances, are caused by inheritance of gene mutations. Table 1 presents the main

neurodegenerative diseases in which a role of NO has been suggested. All of these pathologies exhibit oxidative stress. An increase in nitrotyrosine has been detected in Parkinson's disease [41], amyotrophic lateral sclerosis (ALS) [42], and in Alzheimer's disease [43].

Evidence for NO involvement in pathological human studies

A more intense [heme-NO] signal is observed in the substantia nigra of Parkinson's disease patients when compared with controls [44]. It has been suggested that the toxic role of NO in Parkinson's disease is induced by damage to complex I of the mitochondrial electron transport chain, as observed in patients' substantia nigra [45].

In Huntington's disease, 90% of neurons of the corpus striatum may be deleted, but neurons that stain for NADPH diaphorase (a histochemical marker for nNOS-containing cells) are relatively preserved [46]. It has been suggested that excessive production of NO causes the disease by destroying the neighboring neurons. Other authors have shown that the density of neurons expressing detectable levels of nNOS mRNA was reduced in the striatum of Huntington's disease cases with advanced pathology [47]. Immunocytochemical studies indicate colocalization of a brain-specific protein associated with Huntington's disease called huntingtin-associated protein (HAP1) and nNOS in some neurons [48], suggesting a role of NO in Huntington's disease.

In the brain of Alzheimer's disease patients, nNOS/NADPH diaphorase neurons in the hippocampus are selectively spared [49, 50]. A specific population of these nNOS/NADPH diaphorase neurons in white matter underlying the frontal cortex and the hippocampus are vulnerable in Alzheimer's disease [51]. Both neurofibrillary tangles and senile plaques, the abnormal microscopic structures associated with the disease, express iNOS [52, 53].

A mutation in the copper/zinc SOD (SOD1) gene was found in 20% of patients with familial ALS. One hypothesis suggests that mutations render the copper in the SOD1 active site more accessible to peroxynitrite, allowing the formation of reactive nitrosium-like intermediates with the capacity to nitrate proteins on tyrosine residues [54]. Another suggestion is that mutations increase the peroxidase activity of SOD1, leading to the formation of more hydroxyl radicals from hydrogen peroxide [55, 56]. Abnormal accumulation of neurofilaments occurs in sporadic and familial ALS and in transgenic mice expressing various SOD1 mutations suggesting a possible link between SOD1 and neurofilaments (NFs). In patients, Chou et al. [57] showed colocalization of NOS and SOD1 in the foci of NF accumulation as 'conglomerates' in upper motor neurons and 'axonal spheroids' in lower motor neurons. Peroxynitrite generated from SOD1 and NOS may nitrate tyrosine residues of NFs, thereby altering NF assembly and causing NF accumulation in motor neurons.

Effects of NOS inhibitors in Parkinson's disease and Huntington's disease models

In monkeys and mice, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administrations produce the biochemical and neuropathological changes in the nigrostriatal pathways found in Parkinson's disease. MPTP neurotoxicity is mediated by its metabolite, 1-methyl-4-phenylpyridinium (MPP+), which is formed by the monoamine oxidase (MAO)-B-mediated oxidation of MPTP. MPP+ produces lesions through inhibition of complex I of the mitochondrial electron transport chain. MPTP administration caused a significant increase in 3-nitrotyrosine [58] and specific nitration of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis [59]. Dopamine depletion, loss of substantia nigra dopaminergic neurons, and tyrosine nitration were reduced by 7-nitro indazole (7-NI) in

Table 1. Neurodegenerative diseases in which the role of NO is suggested.

Disease	Mutant gene or genetic susceptibility	Affected region	Characteristic clinical features
Parkinson's	α-synuclein	Substantia nigra	Extrapyramidal symptoms (rigidity, tremor, akinesia)
Huntington's	Huntingtin	Cerebral cortex striatum	Involuntary choreiform movement, cognitive decline
Alzheimer's	Amyloid precursor protein, Presenilins 1 and 2, α-2 macroglobulin, Apolipoprotein E4	Cerebral cortex olfactory bulb, hippocampus	Memory disturbance, dementia
Amyotrophic lateral sclerosis	SOD-1	Motoneurons in spinal cord and brain	Loss of motor activity, paralysis

MPTP-treated mice and monkeys [58, 60, 61]. Although 7-NI can inhibit MAO-B, raising the possibility that this inhibition by 7-NI may also contribute to the observed neuroprotection [62], MPTP neurotoxicity is attenuated by more specific neuronal NOS inhibitors such as S-methylthiocitrulline [63].

Administration of the succinate dehydrogenase inhibitors 3-nitropropionic acid or malonate in rats or mice produce striatal lesions and behavioral abnormalities, characteristics of Huntington's disease, associated with the generation of hydroxyl radicals and 3-nitrotyrosine [64]. Administration of 7-NI, S-methylthiocitrulline, L-NA or N^G-nitro-L-arginine methyl ester (L-NAME) attenuated the toxicity of 3-nitropropionic acid and malonate [63–67].

The beneficial effects of NOS inhibition in Parkinson's and Huntington's disease models strongly suggest a role for deleterious NO production in these pathologies.

NOS-deficient mice

Transgenic nNOS-deficient mice are more resistant to MPTP-induced neurotoxicity than wild-type littermates [61]. Similar results have been obtained against malonate toxicity [68]. These findings directly implicate NO derived from the neuronal isoform in the damage induced by these mitochondrial toxins.

Alzheimer's disease and ALS

Beta-amyloid protein, the main component of senile plaques, can induce iNOS in microglial cells and cytokine-activated astrocytes [69, 70]. Disappointingly, NOS inhibitors have failed to attenuate beta-amyloid toxicity in rat hippocampal cultures [71]. No studies in animal models have yet been performed with NOS inhibitors.

Transgenic mice that overexpress a mutated human SOD1 gene have been shown to develop motor neuron disease that resembles human illness, as revealed by motoneuron loss in the lumbar and cervical spinal regions and a progressive loss of voluntary motor activity in 5–6 months. These mice show lipid and protein oxidative damage to the spinal motor neurons which is associated with disease onset and progression [72]. Cha et al. [73] have reported that reactive astrocytes in spinal cord express nNOS. Whether NO synthase inhibition could play a protective role in ALS remains to be established.

Conclusion

The use of NOS inhibitors and mutant mice lacking each NOS isoform have provided evidence of the injurious effects of NO derived from neuronal or inducible isoforms in neurological diseases. In addition, it appears that neuronal injury is due not only to NO but also to the cooperative effect of NO, superoxide anion, and peroxynitrite. New neuroprotective strategies have been proposed with selective NOS inhibitors for the neuronal (ARL17477) or the inducible (1400W) isoforms, or with compounds combining in one molecule selective nNOS inhibition and antioxidant properties (e.g., BN 80933) in experimental ischemia-induced acute neuronal damage. The efficacy of these new strategies is well established in acute neuronal injury but remains to be determined for more chronic neurological diseases.

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