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NITRIC OXIDE IN THE NERVOUS SYSTEM

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ABSTRACT

Nitric oxide (NO) has only recently been appreciated as a normal biologic substance with a role in signal transduction. It was first identified as endothelial-derived relaxing factor in blood vessels and as the mediator of the tumoricidal and bactericidal actions of macrophages. NO's role as a neural messenger may be even more prominent. Biosynthesis of NO involves oxidation of the guanidine group of arginine with stoichiometric formation of citrulline. NO synthase is one of the most extensively regulated enzymes in biology. In the periphery, NO is a likely transmitter of nonadrenergic, noncholinergic neurons. In the brain, NO neurons mediate action of glutamate acting at *N*-methyl-D-aspartate (NMDA) receptors. Excess release of NO appears to account for a major portion of neural damage following vascular stroke.

INTRODUCTION

The discovery of nitric oxide (NO) as a neurotransmitter and recent evidence that carbon monoxide (CO) may also be a transmitter (1, 2) have radically altered thinking about synaptic transmission. Since NO is a labile free radical, it is not stored in synaptic vesicles as are other transmitters. Instead, it must be synthesized on demand from its precursor L-arginine by NO synthase (NOS), which must be capable of rapid modulation. Not surprisingly, NOS is one of the most regulated enzymes in biology. NO cannot be released by exocytosis but instead simply diffuses from nerve terminals. Rather than bind-

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ing to protein receptors on adjacent cells, it diffuses into them. NO directly interacts with targets that would normally be regarded as second messengers. In contrast to the reversible binding to plasma membrane receptors characteristic of most neurotransmitters, NO forms covalent linkages to intracellular proteins. There may be a substantial number of targets for NO; best characterized is guanylyl cyclase (GC). Inactivation of NO presumably involves diffusion away from target sites. Covalent linkage to small molecules such as superoxide and large proteins may also inactivate synaptically released NO.

One fascinating feature of NO, shared with some neurotransmitters such as peptides, is its involvement in multiple biological processes unrelated to synaptic transmission. NO was first discovered as a substance formed by macrophages, responsible for their ability to kill tumor cells and fungi. Investigations into nitrosamines as carcinogens led to the demonstration that nitrates are produced endogenously. Because mice with a genetic absence of macrophages fail to produce urinary nitrate, the macrophage was identified as a source. The dependence of macrophage production of nitrite upon external arginine led to a characterization of NOS in these cells (3–6).

A second major role of NO is as endothelium-derived relaxing factor. NO was detected as the active metabolite of nitroglycerin and other organic nitrates that dilate blood vessels by stimulating the formation of cyclic GMP through the activation of GC (7, 8). Furchgott & Zawadzki (9) showed that blood vessel relaxation in response to acetylcholine and other substances requires release by the endothelial lining of a labile endothelium-derived relaxing factor, which was subsequently identified as NO (10, 11).

The dramatic properties of NO in macrophages and blood vessels led to suspicion that NO is formed in the brain (12). Definitive evidence for a role of NO in synaptic transmission came from observations that the excitatory neurotransmitter glutamate acting at *N*-methyl-D-aspartate (NMDA) receptors stimulates the formation of NO (13) and that this stimulation is blocked by NOS inhibitors such as nitroarginine and methylarginine, which also block the NMDA stimulation of cGMP in brain slices (13, 14).

NO BIOSYNTHESIS

Though only a single enzyme is involved in NO biosynthesis, the pathway is complex because NOS is an extraordinarily well-regulated enzyme. It oxidizes the guanidino group of L-arginine in a process that utilizes five electrons and gives rise to NO and equal amounts of L-citrulline (15). Numerous attempts to purify NOS failed because enzyme activity was lost rapidly during purification procedures. The discovery that calmodulin is required for NOS activity in the brain enabled us to purify the enzyme to homogeneity (16). We subsequently cloned the cDNA for the brain enzyme neuronal NOS (nNOS) (17).

This step permitted us (18) and others (19, 20) to clone the macrophage form (mNOS). Still other workers cloned endothelial NOS (eNOS) (21–23). Recently cloned inducible forms of NOS from hepatocytes and chondrocytes (24, 25) may mediate the formation of NO in an inducible fashion that has been observed in many tissues.

NOS oxidizes arginine to NO. NOS uses not one electron donor but five. Cloned NOS possesses recognition sites for NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) (Figure 1). Biochemical analysis reveals both FAD and FMN bound to NOS (26). Cytochrome P450 reductase (CPR) is the only mammalian enzyme that also uses NADPH, FMN, and FAD as electron donors. CPR donates electrons to the liver's drug metabolizing cytochrome P450 enzymes. The carboxyl half of NOS displays about 60% amino acid identity to CPR. It is therefore likely that early in evolution CPR donated electrons for NOS and that at some point CPR and NOS fused. NOS also utilizes tetrahydrobiopterin as an electron-donating cofactor (27–29). Recently, we (30) and others (31–33) showed that NOS contains bound heme that reacts with CO to form a species that absorbs at 450 nm. Accordingly,

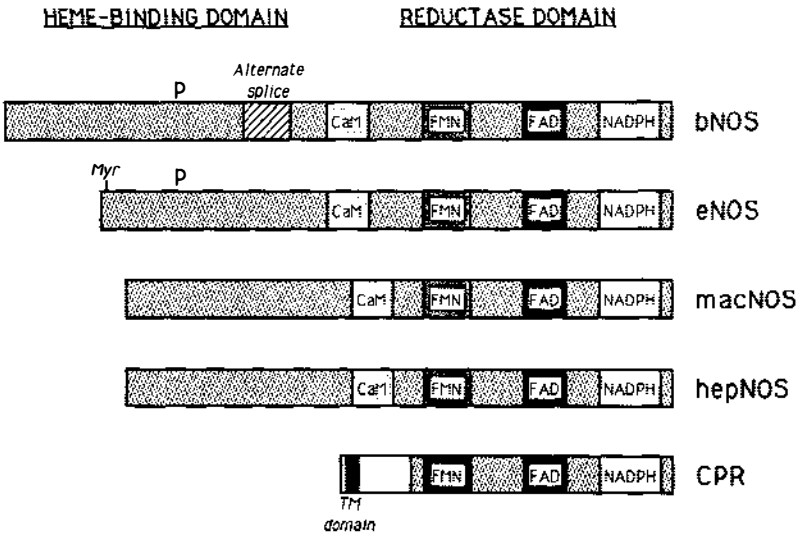

Figure 1 Sequence homologies of NOS isoforms. All NOSs cloned thus far contain regions homologous to cytochrome P450 reductase and coenzyme binding sites that reflect the oxidative mechanism of NO synthesis. Consensus binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), calmodulin (CaM), and heme are conserved for all cloned NOSs. Phosphorylation site (P) for cAMP-dependent kinase is conserved between the neuronal and endothelial forms. The human hepatic NOS contains three consensus sequences for cAMP-dependent phosphorylation. The endothelial NOS has a myristoylation site (M) at its amino-terminus.

NOS has properties of a cytochrome P450 enzyme. The exact mechanism whereby electron donation takes place in NOS is unclear. It is likely that electrons proceed from NADPH to FAD, FMN, and in turn to heme to promote interactions with molecular oxygen, with tetrahydrobiopterin playing some role that is not yet established definitively.

NOS enzymes are constitutive or inducible. The brain form and the endothelial form are regarded as constitutive in that stimuli enhancing NO formation do not elicit new enzyme protein synthesis. Thus, in the brain, glutamate acting at NMDA receptors triggers an influx of calcium that binds to calmodulin to activate NOS. This explains the remarkable ability of glutamate to triple NOS activity in brain slices in a matter of seconds and reflects a novel mode of synaptic information transfer. In blood vessels, stimulation of acetylcholine or bradykinin receptors on endothelial cells activates the phosphoinositide cycle to generate calcium that stimulates NOS. The designation of neuronal NOS as constitutive is not altogether accurate, as recent studies show that new synthesis of nNOS occurs, especially following neuronal damage. For instance, in the spinal cord, lesions of the ventral root lead to the appearance of newly immunoreactive NOS neurons in the spinal cord (34), and nNOS mRNA is induced in dorsal root ganglia following peripheral axotomy (35).

mNOS is not stimulated by calcium despite the fact that it possesses tightly bound calmodulin (36). Calmodulin is so intimately associated with mNOS that it does not dissociate; thus the enzyme is apparently always calcium activated. Macrophages normally possess no detectable NOS protein. Stimuli such as interferon-gamma and lipopolysaccharide (LPS) lead to new NOS protein formation over 2–4 h to produce the NO involved in inflammation. Endotoxin stimulates inducible NOS activity in many animal tissues that do not possess macrophages (37). An inducible NOS cloned from liver cells (24) and chondrocytes (25) may represent a prototype for nonmacrophage-inducible NOS. Because the NO system is simple, inducible NOS may represent a primitive sort of immune system that repels invading microorganisms.

NOS is regulated by phosphorylation. All cloned NOS isoforms possess consensus sequences for phosphorylation by cyclic AMP–dependent protein kinase (PKA). Biochemical studies demonstrate that nNOS is phosphorylated by PKA, protein kinase C (PKC), cyclic GMP–dependent protein kinase (PKG), and calcium calmodulin–dependent protein kinase (CAM-K) (38–41). Though investigators disagree on this point, it appears likely that phosphorylation by all of these enzymes decreases catalytic activity (41). Phosphorylation may interact with other modes of enzyme modulation. For instance, NO stimulates GC to form cyclic GMP which via PKG can inhibit NOS. Calcium-calmodulin directly activates the enzyme, but phosphorylation via CAM-K inhibits. Similarly, calcium activates PKC which in turn inhibits NOS through phosphorylation.

Regulation of mNOS expression derives from transcription factor binding to the regulatory region of the gene. Characterization of the promoter region of macrophage NOS by us (42) and others (43) has revealed multiple regulatory sites. Two distinct regulatory regions exist upstream of the TATA box, which itself is 30 bases upstream of the transcription start site. Region 1, 50–200 base pairs upstream of the start site, contains LPS-related response elements. Region 2, 900–1000 bases upstream, does not directly stimulate NOS expression but instead provides a further enhancement to the 75-fold increase in NOS expression that can be elicited by Region 1. Region 2 contains recognition sites for interferon-gamma-related transcription factors.

This pattern of gene organization can explain certain aspects of inflammation. In sepsis following an overwhelming bacterial infection, LPS is released from bacterial cell walls and circulates throughout the body eliciting inflammatory responses. By contrast, interferon-gamma is released locally, augmenting inflammatory responses only in certain populations of cells close to the site of interferon-gamma release. Thus, the local elaboration of interferon-gamma by infiltrating lymphocytes primes macrophages to give a maximal response to circulating LPS. In this way, maximal production of NO occurs primarily in cells at the site of the infiltrating lymphocytes—cells located in a position to attack the invading microorganisms without damaging other tissue.

NO in Neurotransmission and Neural Development

Localization of neuronal NOS has helped clarify many of its functions. In areas such as the cerebral cortex, hippocampus, and corpus striatum, NOS neurons compose only 1–2% of all neuronal cells; scattered in no obvious pattern, they make up medium-to-large aspiny neurons (44). By contrast, in the cerebellum NOS occurs in a high proportion of certain cell types. It occurs in almost all granule and basket cells but in no Purkinje cells. The mode of NO action in specific synaptic systems is best exemplified in the cerebellum. Glutamate augments cyclic GMP concentrations in Purkinje cells, which receive input from granule and basket cells. Granule and basket cells possess NMDA receptors. Stimulation by glutamate thus triggers formation of NO, which diffuses to the Purkinje cells to activate GC. Whether GC is a universal target for NO is not clear. If NO transmission exclusively utilized GC and if all GC in the brain were associated with NO, then GC and NOS localizations would be essentially the same. However, their localizations differ markedly, whereas localizations of GC and heme oxygenase-2 (the CO-synthesizing enzyme) are similar (1).

Clues into neurotransmitter function often come from co-localization with other transmitters. The work of Hokfelt (45) has revealed that virtually all neurons in the brain contain more than one neurotransmitter. In the cerebellum,

NOS occurs in the glutamate-containing granule cells and in basket cells that contain GABA. Some but not all the cerebral cortical NOS neurons also contain GABA. In the corpus striatum, NOS neurons stain for somatostatin and neuropeptide Y. On the other hand, in the pedunculopontine nucleus of the brain stem, NOS neurons stain for choline acetyltransferase but lack somatostatin and neuropeptide Y (46).

NO influences neurotransmitter release. NOS inhibitors block the release of transmitters in various systems. In synaptosomes of the brain, neurotransmitter release evoked by stimulation of NMDA receptors is blocked by nitroarginine, whereas release elicited by potassium depolarization is unaffected (47). Glutamate apparently acts on the NMDA receptors of NOS terminals to stimulate the formation of NO. NO diffuses to adjacent terminals and increases neurotransmitter release. Thus blockade of NO formation inhibits release. On the other hand, potassium depolarization triggers release from all terminals such that effects of NO are not detectable.

PC-12 cells develop neuronal properties when stimulated with nerve growth factor. Acetylcholine release in response to depolarization in PC-12 cells is markedly stimulated after about 8 days of nerve growth factor application (48, 49). NOS staining and catalytic activity are not apparent in untreated cells but develop 8 days after nerve growth factor stimulation, coincident with enhancement of transmitter release. Acetylcholine and dopamine release from PC-12 cells is blocked by NOS inhibitors and reversed by L-arginine (47).

The peripheral autonomic nervous system has provided the most definitive evidence that NO is a neurotransmitter. In our initial mapping studies we observed NOS neurons in the myenteric plexus from the esophagus to the rectum (46, 50). Physiologic depolarization of these neurons elicits relaxation of smooth muscle associated with peristalsis. Blockade of this process by NOS inhibitors indicates that NO is the transmitter (51–54).

Further evidence that NO functions in the peripheral autonomic innervation of the gastrointestinal system comes from mice in which nNOS has been "knocked out" by homologous recombination (55). These mice display markedly enlarged stomachs and hypertrophy of the pyloric sphincter (55). Similar abnormalities occur in the human disease infantile hypertrophic pyloric stenosis. Histochemical studies of stomachs of such patients reveal an absence of NADPH diaphorase (NDP) staining in the pyloric region (56). This observation may help to account for the symptoms of the disease. The relaxing effects of NO would normally facilitate propulsion of food through the pyloric sphincter, just as NO is presumably involved in the relaxation phase of peristalsis throughout the gut. Absence of NO would account for an abnormally tight sphincter.

nNOS is found in the endothelial layer of blood vessels, but it also occurs in autonomic nerves in the outer, adventitial layers of some vessels, especially

larger ones (50). In the retina (57) and cerebral cortex (58), these neurons arise from cells in the sphenopalatine ganglia at the base of the skull. About 40% of these NOS neurons contain the neuropeptide vasoactive intestinal polypeptide (VIP), which may function as a cotransmitter regulating blood vessel relaxation. In the penis, NOS neurons are particularly prominent in cavernous nerve processes innervating the deep cavernosal arteries and sinusoids of the corpora cavernosae, the erectile bodies of the penis whose engorgement with blood leads to penile erection (59). Electrical stimulation of the cavernous nerve in intact rats produces prominent penile erection that is blocked by low doses of intravenously administered NOS inhibitors (59). Nerve stimulation–induced relaxation of isolated corpus cavernosum stips is also blocked by NOS inhibitors (60). These findings establish NO as the transmitter of neurons regulating penile erection.

In the adrenal gland, NOS occurs in ganglion cells and fibers in the adrenal medulla (50), where it may regulate blood flow (61). The function of the prominent NOS neuronal fibers in the posterior pituitary has not been clarified (50).

NO may participate in long-term potentiation (LTP), which may serve as a model of learning and memory. While LTP can be demonstrated at many synapses, the best-studied occurs in the hippocampus between terminals of the Schaffer collaterals and CA1 pyramidal cells. LTP requires the involvement of both presynaptic and postsynaptic elements. Enhancement of transmitter release by Schaffer collateral terminals is thought to require release by pyramidal cells of a "retrograde messenger." NO had been advanced as a candidate for the retrograde messenger role, because NO inhibitors interfere with the establishment of LTP, as does hemoglobin, which binds NO (62–65). However, immunohistochemical studies fail to reveal any nNOS in pyramidal cells (44). Moreover, LTP can readily be demonstrated in hippocampal slices of mice in which nNOS has been knocked out by homologous recombination (66). Even more perplexing is the fact that NOS inhibitors continue to block LTP in the nNOS knock-outs. A solution to this riddle may come from immunohistochemical studies showing that endothelium of blood vessels is not the only site of eNOS occurrence. Instead, we observe high eNOS densities in pyramidal cells of the hippocampus (67). In nNOS knock-out, eNOS localizations in the pyramidal cells and other neurons and in blood vessel are normal (66). While definitive evidence awaits studies in eNOS knock-outs, NO derived from eNOS in pyramidal cells may well be important to LTP in the hippocampus.

The fact that NO is formed in neurons by two discrete NOS isoforms derived from different genes may help to clarify the functions of NO in the brain. Differences in localization of the two enzymes may be illuminating. Both forms of NOS occur in the olfactory bulb, caudate-putamen, supraoptic nucleus, and

cerebellum. However, only eNOS is concentrated in the hippocampus, both in pyramidal cells of the CA1 region and in granule cells of the dentate gyrus. By contrast, nNOS occurs only in small GABA-containing interneurons and pyramidal cells of the subiculum. In the olfactory bulb the two enzymes are differentially localized: eNOS occurs in neurons of the internal granule cell layer and neuropil of the glomerular and external plexiform layers, while nNOS is found in individual neurons of the glomerular layer. nNOS is less prominent than eNOS in neuropil in the external plexiform layer, though in the internal granule cell layer its localizations are similar to those of eNOS. Neither eNOS nor nNOS occurs in mitral cells. In the caudate-putamen, moderate amounts of eNOS occur in small-to-medium spiny neurons, whereas nNOS is found in medium-to-large aspiny neurons. Cerebellar eNOS is less prominent in granule cells than is nNOS, and neither occurs in the Purkinje cells. nNOS (but not eNOS) is evident in the superior and inferior colliculi, the bed nucleus of the stria terminalis, and the hypothalamus.

nNOS neurons stain prominently for NADPH diaphorase (NDP), whereas in most studies NDP is never detected in cells (such as pyramidal cells of the hippocampus) that contain high levels of eNOS. Higher concentrations of glutaraldehyde reveal eNOS as well as nNOS staining (67). Glutaraldehyde fixation differs from formaldehyde fixation, which is conventionally used for NDP, in that it is irreversible and produces many more intramolecular cross-links than formaldehyde. Since eNOS is myristoylated and largely membrane associated (68, 69), glutaraldehyde-containing fixatives should better preserve the active form of eNOS by crosslinking it to other components of the plasma membrane.

NO may play a role in development of the nervous system. Our immunohistochemical studies reveal transient expression of nNOS in discrete areas of the developing rat nervous system (70). In the brain, transient NOS expression occurs selectively in the cerebral cortical plate, especially at embryonic days E15–19 in which the majority of the cells in the plate stain and their processes pass to the thalamus. The innervation gradually decreases after birth and is absent in adults. Similarly, in the olfactory epithelium nNOS occurs prominently in neurons from E15 to early postnatal life. In embryonic sensory ganglia virtually all neuronal cells are nNOS positive, whereas in adult life only 1% express NOS.

The function of NO in the developing nervous system is unclear. NO may be trophic for developing neurons. Alternatively, it may help to elicit the programmed cell death in which up to 50% of mammalian neurons die during development. The limited amount of evidence now available suggests that NO may be involved in cell death. For instance, NO-generating agents cause a collapse of neuronal growth cones in regenerating dorsal root ganglion cells (71). Although originally nNOS was not thought to be inducible, various forms

of nerve lesion increase expression of nNOS. Following peripheral axotomy, the percentage of NOS-positive cells in sensory ganglia increases markedly, though not to levels evident in the embryonic ganglia (35). In the brain, lesions of the medial forebrain bundle and mammillothalamic tract elicit nNOS staining in cell bodies of the lesioned fibers; this staining persists up to 5 months, suggesting a neuroprotective role of nNOS action (72). Kalb & Agostini (73) detected transient expression of NOS in ventral horn neurons that contact motor neurons in the first postnatal week of life; nitroarginine treatment blocked the development of sciatic motor neurons. Studies in the spinal cord suggest that NO formed by nNOS induced following lesions is responsible for cell death. Thus, following ventral root avulsion or spinal cord transsection, motor neurons display pronounced nNOS staining (34). The cells that display the augmented staining ultimately die (74). Moreover, treatment of these animals with the NOS inhibitor nitroarginine prior to ventral root avulsion protects the cells from death (74).

A Role for NO in Neurotoxicity

Abundant evidence implicates NO as a mediator of neurotoxicity, especially in response to glutamate. Following vascular stroke, large amounts of glutamate are released that elicit neurotoxicity via NMDA receptors, a finding supported by the protective effects of NMDA antagonists (75, 76). NMDA receptor stimulation augments NOS activity, but NOS neurons are resistant to NMDA neurotoxicity following NMDA stimulation of cortical cultures, both in stroke and in Huntington's and Alzheimer's diseases (77–81). NO released from NOS neurons in response to NMDA stimulation appears to kill adjacent neurons: NOS antagonists prevent neurotoxicity in cortical culture, as does removal of arginine from the medium and treatment with flavoprotein and calmodulin inhibitors (82, 83). Low doses of nitroarginine block neural damage following middle cerebral artery occlusion in mice, rats, and cats (84–88). High doses of NOS inhibitors may exacerbate this damage, presumably through decreased cerebral blood flow (89, 90). NO neurotoxicity may result from the formation of peroxynitrite by the combination of NO with superoxide. Such peroxynitrite should lead to the formation of nitrotyrosine in proteins. Using an antibody to nitrotyrosine, J Beckman (personal communication) has demonstrated dramatic staining co-localized with infarcted tissue following middle cerebral artery ligation in rats. Particularly persuasive evidence for a role of NO in stroke damage comes from the observation (90a) that stroke damage is reduced by about 40% in nNOS knock-out mice.

NO may also mediate neurotoxicity in AIDS dementia, a puzzling condition in which the HIV virus is detected at negligible levels in neurons. It is thought that the gp120 coat protein mediates toxicity, as picomolar concentrations kill neurons in primary cerebral cortical cultures in a fashion absolutely dependent

upon the action of glutamate through NMDA receptors (91). We observed that this toxicity requires NO because it is absent in arginine-free medium and blocked by NOS inhibitors (92). Because gp120 toxicity requires the presence of macrophages and/or astrocytes whose released cytokines and arachidonic metabolites can potentiate NMDA receptor currents (93), we suspect that these other mediators synergize with glutamate to trigger NO formation.

Further support for a role of NO in neurotoxicity comes from studies using protective agents that indirectly block NO formation. Gangliosides are neuroprotective in both animals and humans with spinal cord damage (94–97). They bind calmodulin (98, 99), inhibit NOS activity, and prevent NMDA toxicity in neuronal cultures in proportion to their affinity for calmodulin and NOS inhibition (100). Immunosuppressants, such as FK506 and cyclosporin A, inhibit the calcium-activated phosphatase calcineurin by binding to the immunophilins cyclophilin and FK-binding protein (FKBP); the drug-immunophilin complex then binds to calcineurin. NOS is a calcineurin substrate, as phosphorylated NOS levels are increased by FK506 and cyclosporin A (101). Because phosphorylated NOS is catalytically inactive, treatment with immunosuppressants is equivalent to treatment with NOS inhibitors. Both FK506 and cyclosporin A block NMDA neurotoxicity in low concentrations (101). In liver transplant patients, the incidence of global cerebral ischemia is much lower in patients receiving FK506, which penetrates into the brain, than in patients receiving cyclosporin, which does not (102). The neuroprotective effects of FK506 were demonstrated recently in focal cerebral ischemia (102a). In rats with middle cerebral artery occlusion, cortical damage was significantly reduced by FK506 treatment (102a).

How does NO exert its neurotoxic actions? In mediating physiologic synaptic transmission, the best-established target of NO is GC. Because GC inhibitors do not block neurotoxicity and because 8-bromo-cyclic GMP, which penetrates readily into cells, is not neurotoxic, other targets must exist for neurotoxicity (103).

Many other NO target molecules have been discovered (Table 1). NO combines with nonheme iron in numerous enzymes such as NADH-ubiquinone oxidoreductase, NADH-succinate oxidoreductase, and *cis*-aconitase, all iron-sulfur enzymes (37, 104). NO binds to the iron in ferritin, the iron storage protein, liberating iron that can cause lipid peroxidation (105). NO also binds to the iron of ribonucleotide reductase to inhibit DNA synthesis (106, 107). Recent evidence indicates a role of NO in regulating iron metabolism. Iron metabolism is influenced posttranscriptionally by mRNA-protein interactions between iron regulatory factors (IRF) and iron-responsive elements (IRE) in the untranslated regions of mRNA for the erythroid form of 5-aminolevulinate synthase, the transferrin receptor, and ferritin (108, 109). IRF is identical to cytosolic aconitase, which is converted to IRF when it loses an iron and is

transformed from a protein with aconitase catalytic activity to a protein that binds IRE. By binding to iron, NO stimulates the IRE-binding function of IRF while diminishing its cytosolic aconitase activity (110, 111). Stimulation of macrophages with LPS or interferon-gamma to produce NO activates IRF function (110, 111). NMDA acting through NO similarly stimulates IRF (112) (Figure 2). IRF occurs in discrete neuronal populations, suggesting a selective synaptic function (112). Though IRF and NOS do not co-localize fully, structures enriched in IRF, such as granule cells of the cerebellum and the olfactory bulb and the dentate gyrus of the hippocampus, also have high densities of NOS. IRF may be sensitive to oxidants other than NO and may couple cellular responses to oxidative states. Influences of NO on IRF might regulate the role of fatty acids in cellular energy dynamics. By diminishing cytosolic aconitase activity, NMDA synaptic transmission would make more citrate available for cytosolic citrate lyase, which converts citrate to acetylCoA and oxalacetate. Cytosolic acetylCoA is prominently incorporated into fatty acids that provide an energy reservoir for neuronal function during states of oxidative stress.

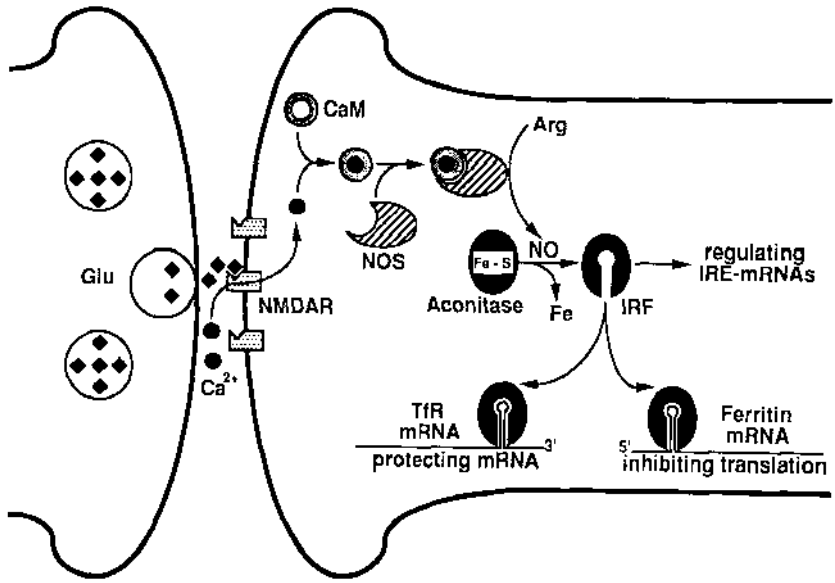

Figure 2 NO activation of IRF in regulation of cellular iron homeostasis. Glutamate (Glu) binds to *N*-methyl-D-aspartate receptors (NMDAR) and causes calcium influx. Elevated intracellular calcium activates calmodulin (CaM), which in turn activates NOS to produce NO from arginine (Arg). NO removes iron (Fe) from cytosolic aconitase, converting it to iron-responsive factor (IRF). IRF regulates iron metabolism through binding to iron-responsive elements (IRE) in a number of mRNAs that encode proteins related to iron transport and storage. If the IRE is located at the 3′ end of mRNA [e.g. transferrin receptor (TfR) mRNA], IRF binding stabilizes the mRNA and increases translation. If IRE is located at the 5′ end of mRNA (e.g. transferrin mRNA), IRF binding prevents the translation.

Table 1 NO targets[a]

Interaction sites or modification types	Target molecules	Functional consequences	References
Heme	Soluble guanylyl cyclase	Increased cGMP	7
	Cyclooxygenase I & II	Increased prostoglandin E_2	130–133
	Cytochrome P450	Impaired detoxification	134, 135
Nonheme-iron	Cytosolic aconitase	Inhibition of glycolysis, activation of IRF to regulate iron metabolism	110–112
	Mitochondrial aconitase	Inhibition of glycolysis	37, 104
	Complex I and II	Inhibition of respiratory chain	37, 104
	Ferritin/transferrin	Iron loss and lipid peroxidation	105
	Ribonucleotide reductase	Inhibition of DNA synthesis	106, 107
Nitrosylation	NMDA receptor	Block of Ca^{2+} influx	136
	Protein kinase C	Inhibition of phosphorylation	137
	GAPDH	Inhibition of glycolysis, enhance ADP-ribosylation	115–117
	Ca^{2+}-dependent-K^+-channel	Activation, vasorelaxation	138–140
	G proteins	Activation, causing NF-κB translocation	141
	Tyrosine phosphatase	Activation of $p56^{lck}$	142
	Albumin	NO carrier activity	143
	t-PA	Vasodilation and antiplatelet activities	113
	Glutathione	Activation of the hexose monophosphate shunt	144
Superoxide	Zn, Cu-superoxide dismutase	Tyrosine nitrations	145
($ONOO^-$ formation)	α-Tocopherol in LDL	Oxidation	146
	DNA strand break	PARS activation	124, 147
	GAPDH	Inhibition of glycolysis	122
Deamination	DNA	Mutations and strand breaks, PARS activation	124, 148

[a] Abbreviations: IRF, iron-responsive factor; complex I & II, NADH-succinate oxidoreductase and NADH-ubiquinone oxidoreductase; NMDA, *N*-methyl-D-aspartate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor κB, a transcription factor; t-PA, tissue-type plasminogen activator; $ONOO^-$, peroxynitrite; LDL, low-density lipoproteins; PARS, poly(ADP-ribose) synthetase.

NO can stimulate the S-nitrosylation of numerous proteins (113, 114) as well as the auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (115–117). The covalent modification of GAPDH by NAD was initially characterized as a mono-ADP-ribosylation. Kots et al (115) and Dimmeler et al (118) determined that radioactivity could be incorporated into GAPDH from [adenylate-^{14}C]NAD but not from [nicotinamide-^{14}C]NAD, suggesting that

ADP-ribose, but not the nicotinamide moiety, was transformed to GAPDH. However, McDonald & Moss (119), under different conditions, observed the full NAD molecule covalently linked to GAPDH through an NO-dependent thiol intermediate. The exact chemical nature of the modification may be more complicated than what was originally conceived. Recent isoelectric focusing gel analysis resolves at least three species of NO-enhanced NAD-modification of GAPDH. The major labeling spots comigrate with ADP-ribosylated GAPDH (120). The exact species of NAD-modified GAPDH in the intact cells is not yet established.

NO-enhanced ADP-ribosylation of GAPDH inhibits its enzymatic activity (116). Although the stoichiometry of ADP-ribosylation of GAPDH is generally less than 10% (115, 117), inhibition of GAPDH by NO is usually more than 50% (121). Apparently, the direct NO modification of cysteine 149 at the catalytic center of GAPDH accounts for the inhibition (121). This NO-modification of GAPDH can also be mediated through peroxynitrite (122).

There is accumulating evidence that endogenous NO production enhances ADP-ribosylation of GAPDH in vivo. Dimmeler et al (123) showed a correlation of interleukin-1β-induced NO production with the increasing of GAPDH ADP-ribosylation in RINm5F cells. In LPS-treated mice, GAPDH activity is reduced in heart and spleen. Thus, NO-induced GAPDH inhibition may contribute to NO cytoxicity by impairing energy production (123).

The best-established candidate for mediation of neurotoxicity by NO is poly (ADP-ribose) synthetase (PARS). Cell death appears to follow NO-mediated DNA damage, which stimulates PARS activity (124). PARS is a nuclear enzyme that utilizes NAD as a substrate to attach 50–100 ADP-ribose units to nuclear proteins such as histones and PARS itself (125). NO stimulates poly (ADP-ribosyl)ation of PARS in brain tissue (124). Moreover, NMDA neurotoxicity in cortical cultures is blocked by PARS inhibitors in proportion to their potency in inhibiting PARS (124). Thus NO neurotoxicity may commence with NO damage to DNA, which activates PARS, depleting cells of NAD and ATP, because four molecules of ATP are consumed in NAD regeneration. While massive stimulation of PARS kills cells by energy depletion, PARS activation by lesser degrees of DNA damage presumably facilitates DNA repair.

PARS activation by damaged DNA enhances DNA repair by relaxing chromosomal structure through poly(ADP-ribosyl)ation of histones, high-mobility group proteins, and nuclear matrix proteins; and by coordinating, through poly(ADP-ribosyl)ating, key enzymes involved in DNA metabolism such as topoisomerase I and Ca^{2+},Mg^{2+}-dependent endonuclease. Ribonucleotide reductase, a rate-limiting enzyme for DNA synthesis, is inhibited by NO (106, 107). Thus, DNA repair is delayed owing to a short supply of deoxyribonucleotides so that PARS activation is prolonged. Meanwhile, the constitutive

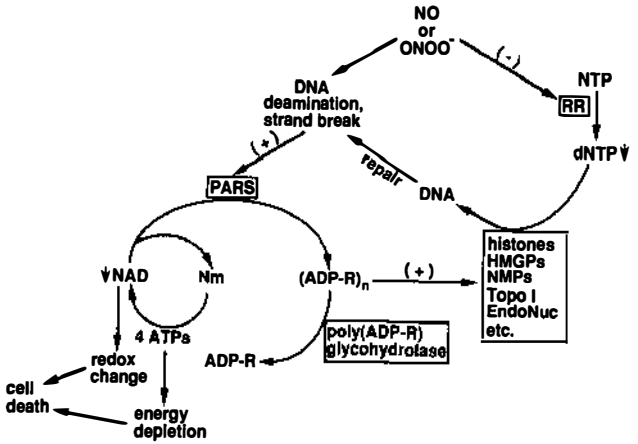

Figure 3 NO-mediated neurotoxicity. DNA damaged by NO or peroxynitrite ($ONOO^-$) activates poly(ADP-ribose) synthetase (PARS), which coordinates DNA repair through poly(ADP-ribosyl)ating itself and other proteins involved in DNA metabolism, such as histones, high-mobility group proteins (HMGPs), nuclear matrix proteins (NMPs), topoisomerase I (Topo I), and Mg^{2+}, Ca^{2+}-dependent endonuclease (EndoNuc). NO inhibition of ribonucleotide reductase (RR) diminishes the deoxyribonucleotide triphosphate (dNTP) supply for DNA synthesis. The delayed DNA repair prolongs PARS activation. At the same time, constitutive poly(ADP-ribose) glycohydrolase rapidly degrades poly(ADP-ribose). Four ATPs are needed to resynthesize nicotinamide adenine diphosphate (NAD) from nicotinamide (Nm). PARS and poly(ADP-ribose) glycohydrolase form a futile cycle that decreases NAD, depletes cellular energy, and ultimately leads to cell death.

poly(ADP-ribose) glycohydrolase is rapidly degrading poly(ADP-ribose). Together, PARS and poly(ADP-ribose) glycohydrolase form a futile cycle to deplete NAD. Because it takes four ATPs to resynthesize one NAD from nicotinamide, a sustained consumption of NAD will deplete ATP, and the resultant drop of cellular energy level may be lethal (Figure 3). PARS inhibitors also protect against NMDA toxicity in mouse fetal cortical culture (126) and against NO toxicity in hippocampal slices (127).

NO activation of PARS is not restricted to neurotoxicity. Inhibition of insulin secretion associated with damage to islets of Langerhans is induced by interleukin-1β and tumor necrosis factor-α derived from activated macrophages, which may contribute to type I diabetes (128). PARS inhibitors can rescue NO-mediated damage of islet cells (129).

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