

SY 2. ROLES OF NITRIC OXIDE IN HEALTH AND DISEASE: CVS AND CNS ASPECT

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INTRODUCTION

The nomination of nitric oxide (NO) as *Science's* "Molecule of the Year 1992" was a milestone for a seemingly common and unstable gas known as a component of cigarette smoke and an atmospheric pollutant. It was then justified by the discovery of myriad effects of NO as a chemical messenger in the cardiovascular system (CVS), immune system, peripheral nervous system (PNS) and central nervous system (CNS). Undoubtedly, NO has a wide-ranging role in health and disease. While many of its effects are well known, there remains much more to explore and to learn about the interactions of this fascinating molecule in physiological and pathophysiological processes.

This article emphasizes potential roles of NO in the CVS and CNS and serves as a guide to the world of booming research with NO and its synthesizing enzyme, NO synthase (NOS). In this respect, most of the content is a simplified and informal overview of NO's roles in health and disease.

Nitric oxide is a soluble gas continuously synthesized by the endothelium. This substance has a wide range of biological properties that maintain vascular homeostasis, including modulation of vascular dilator tone, regulation of local cell growth, and protection of the vessel from injurious consequences of platelets and cells circulating in blood. A growing list of conditions, including those commonly associated as risk factors for atherosclerosis such as hypertension and hypercholesterolemia, are associated with diminished release of nitric oxide into the arterial wall either because of impaired synthesis or excessive oxidative degradation. Diminished nitric oxide bioactivity may cause constriction of coronary arteries during exercise or during mental stress and contribute to provocation of myocardial ischemia in patients with coronary artery disease. Additionally, diminished nitric oxide bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, the precursor of the atherosclerotic plaque. Numerous therapies have been investigated to assess the possibility of reversing endothelial dysfunction by enhancing the release of nitric oxide from the endothelium, either through stimulation of nitric oxide synthesis or protection of nitric oxide from oxidative inactivation and conversion to toxic molecules such as peroxynitrite.

Eventually, the discovery of NO as a uniquely diffusible and reactive molecular messenger in the vascular system motivated searches for NO biosynthesis throughout the body. NO was then found in abundance in the immune, central and peripheral nervous systems. Indeed, NOS, the enzyme that produces NO from L-arginine, occurs at higher levels in brain than in any other tissue. Intensive studies over the past 10 years have determined that NO mediates diverse physiological functions associated with neurons and

other excitable cells. In the peripheral nervous system, NO acts much like a classical neurotransmitter in regulating gastrointestinal motility, regional blood flow, and neuroendocrine function. In the brain, NO acts as a neuromodulator to control behavioral activity, influence memory formation, and intensify responses to painful stimuli. Furthermore, NO biosynthesis in excitable tissues is not restricted to neurons. Recent studies have identified skeletal muscle as a major source for NO in the body where NO regulates both metabolism and muscle contractility.

NO biosynthesis in excitable tissues is regulated by increases in intracellular calcium, which activate NOS through the enzyme's dependence upon calmodulin. Although small amounts of NO synthesized during neural and skeletal muscle activity mediate physiological functions, excess NO production can mediate tissue injury. For example, large amounts of NO produced during periods of cerebral ischemia mediate neuronal injury in various forms of stroke. Similar NO-mediated damage may account for neurodegeneration in other conditions as well, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease. NO signaling is also perturbed in various muscle diseases, particularly in Duchenne muscular dystrophy, and these derangements may contribute to the disease processes. Therefore, pharmacological regulation of NO synthesis offers an important strategy for treatment of neurodegenerative and muscle diseases.

CHEMICAL CONSIDERATION OF NO

The signal molecule NO is synthesized on demand by constitutively expressed NOS (cNOS), namely, endothelial NOS (eNOS) and neuronal NOS (nNOS), for short periods of time (seconds to minutes). The killer molecule NO is synthesized by an inducible NOS (iNOS) that, once expressed, produces NO for long periods of time (hours to days). According to calculations, the major differences between cNOS and iNOS activities do not reside in the concentrations of NO generated per enzyme, but rather in the duration of NO produced. In addition, the iNOS protein content in fully activated cells may be higher than the cNOS content. Thus, cytotoxicity usually correlates with the product of iNOS and not with the product of the two cNOS (with possible exceptions in brain injury). Thus, regulated pulses versus constant unregulated NO synthesis differentiates between the messenger and the killer properties of NO.

Although being a radical, NO has quite a long life in biological environments, depending on its own as well as concentrations of oxygen and/or other components of the solvent. In addition, reaction with glutathione, or proteins containing reduced cysteine moieties, yields S-nitrosothiols, which are more or less unstable (half-lives: minutes to hours) and decompose, again yielding NO. These can thus be regarded as NO-storage compounds. It is, therefore, impossible to determine exactly the life span of NO in biological systems.

Concerning the cytotoxic effects of NO, one must ask if the effect is really due to nitrogen monoxide. Concerning the physiological signaling function of NO, we can be certain that we deal with NO as the target molecule. The soluble guanylate cyclase (sGC) is activated by NO with an apparent K_M in the nanomolar range and not by other related compounds. Concerning the toxic effects of NO, the picture is less clear, especially under the aerobic conditions of cellular life. In addition, activated cells like macrophages, neutrophils, or endothelial cells secrete reactive oxygen intermediates, like H_2O_2 , $O_2^{\cdot-}$, OH^{\cdot} , and singlet oxygen (1O_2), thereby complicating the local environmental situation. Reaction of NO with H_2O_2 yields 1O_2 and a cooperative toxic action of NO and H_2O_2 has been

demonstrated. $O_2^{\cdot -}$ and NO may react to form the peroxynitrite anion ($ONOO^-$), a strong oxidant with a half-life of less than a second. However, macrophage generation kinetics of $O_2^{\cdot -}$ is different from kinetics of NO generation and the amount of $ONOO^-$ generated extracellularly by activated macrophages and endothelial cells *in vitro* appears to be very small. In addition, NO seems to inhibit $O_2^{\cdot -}$ production by activated neutrophils by either decreasing NADPH activity or assembly. The NO oxidation or reduction products, NO^+ and NO^- , respectively, probably do not play a significant role in biological systems, as NO cannot be oxidized to NO^+ *in vivo*. Additionally, both NO^+ and NO^- are highly reactive and would disappear rapidly if formed. In contrast, reaction pathways of NO with molecular dioxygen yielding nitrogen dioxide (NO_2), the peroxynitrite radical ($ONOO^{\cdot}$), dinitrogen trioxide (N_2O_3), and/or other compounds are likely to occur *in vivo*. In conclusion, NO secreted by activated cells appears to be a complex "cocktail" of substances. Nevertheless, synthesis of true NO is the necessary first step for creating these compounds.

NO may react with proteins and nucleic acids. In addition to binding to heme groups, e.g., of guanylate cyclase, hemoglobin, and cytochrome c oxidase, NO theoretically may react with nucleophilic centers like sulfur, nitrogen, oxygen, and aromatic carbons. The prime target for covalent binding of NO to functional groups in proteins under physiological conditions in the presence of oxygen is the SH group. Tryptophan is the only noncysteine residue that undergoes nitrosation by NO. However, this reaction is about 10-fold slower than S-nitrosylation and thus cannot compete favorably with cysteine in a spontaneous nitrosation reaction. Deamination reactions of protein amino groups by NO have been proposed, but not shown. N-nitrosation of secondary NH_2 groups, e.g., of lysin moieties, and subsequent deamination reactions could not be observed under physiological conditions, even when high NO concentrations were used. Nitration of tyrosine residues by NO has been proposed also, but could not be confirmed. NO has been shown to N-nitrosylate primary arylamines of nucleotides and subsequent hydrolysis yields deaminated nucleotides. However, the selectivity ratio is at least 10^6 times greater for sulfhydryl-containing peptides than for exocyclic amines of DNA bases.

MOLECULAR TARGETS OF NO

Prior to discussing potential targets of NO within cells, an unsolved mystery must be discussed. While active cNOS is a membrane-bound protein, active iNOS is located in the cytosol. How does the iNOS product NO escape from the cytosol of the donor cell without reacting with the many intracellular targets and without causing damage to the effector cell itself? To prevent unintentional reactions, either special intracellular transport routes for NO, e.g., in acidic compartments, or, alternatively, harmless NO-transport molecules must exist. Several intracellular transport forms of NO have been proposed but have not been proven.

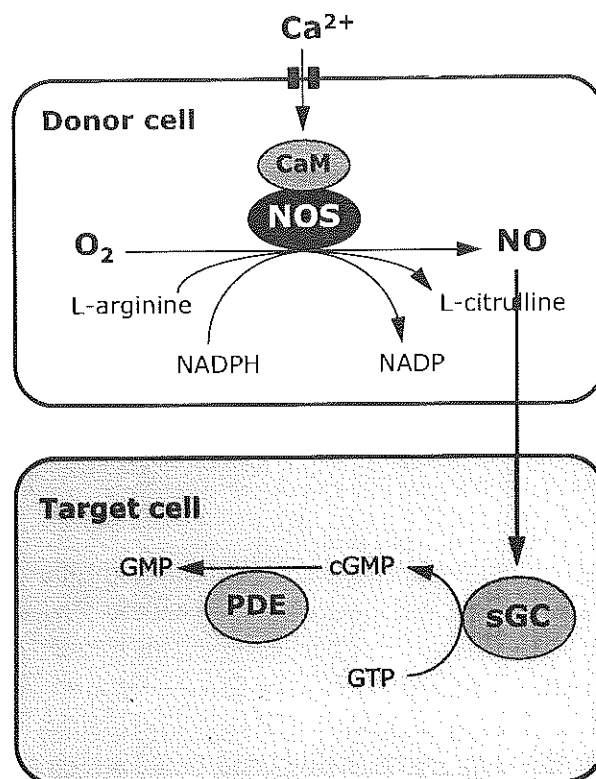


Figure 1. General mechanism of NO–cGMP signal transduction. Biological activity in the NO donor cell leads to the influx of Ca^{2+} , which stimulates via calmodulin (CaM) and the NOS enzyme. NOS catalyses the conversion of arginine to citrulline, which is formed stoichiometrically with NO. This reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. In the target cell, NO binds to a heme moiety in soluble guanylate cyclase (sGC), resulting in the stimulation of the enzyme and consequent elevation in cGMP concentration. cGMP is efficiently metabolized by phosphodiesterases (PDE).

After being secreted by the donor cell in a way not yet understood, NO then diffuses to a target cell located nearby and hits its plasma membrane. O_2 and NO closely resemble each other in diffusability and fluid-phase membranes are no diffusion barriers for NO. There are several targets for NO at the surface or within the plasma membrane, e.g., transport and signaling proteins and surface receptors among others. To date, reactions of NO with surface receptors leading to a chemical modification of these receptor proteins have not been reported, with the exception of the neuronal NMDA receptor-channel complex. However, the proposed mechanism, inhibition of the redox modulatory site of this complex via S-nitrosylation, has been recently questioned. Permeability to K^+ , Na^+ , H^+ , and Ca^{2+} is the most important factor controlling the proton motive force and the membrane potential. A number of publications show activation of K^+ channels as well as $\text{Na}^+\text{-K}^+\text{-ATPase}$ in vascular smooth muscle cells by low concentrations of NO. On the other hand, high NO concentrations inhibit neuronal $\text{Na}^+\text{-K}^+\text{-ATPase}$, K^+ and Ca^{2+} channels in islet cells, and $\text{H}^+\text{-ATPase}$ in brain synaptic vesicles. In addition, NO has been shown to depolarize the membrane potential of a tumor cell line. In conclusion, NO changes ion currents through the plasma membrane and thus alters the plasma membrane potential.

After diffusion into the target cell, NO can inhibit SH-dependent enzymes via S-nitrosylation. Notably, inhibition of creatine kinase, affecting local ATP regeneration, might contribute to NO-mediated cell injury. A second mechanism of NO-mediated enzyme inhibition has been found with certain heme-containing enzymes, e.g., cytochrome P450 isoenzymes. After formation of a heme-NO adduct, a secondary oxygen-dependent reaction takes place which results in an irreversible nitration of a tyrosine in the active-site pocket. NO also mediates Fe^{2+} release from target cells, destroying Fe-S clusters in enzymes, like the citric acid cycle enzyme aconitase or ferrochelatase, which catalyze the insertion of Fe^{2+} into protoporphyrin. Other intracellular targets for NO are proteins containing zinc fingers, ring fingers, and the LIM motif, respectively. A common feature of these structures is Zn^{2+} complexed, sometimes together with histidine imidazol nitrogens, by cysteine sulfur ligands. This creates tertiary protein structural "finger" domains that specifically bind to DNA or RNA sequences. Many of these proteins are involved in transcription, replication, recombination, or restriction. We could show that NO mediates Zn^{2+} release *in vitro* from the Zn^{2+} storage protein metallothionein and inhibits the DNA-binding activity of the zinc finger transcription factor LAC9. Others found NO-mediated inhibition of protein kinase C, which contains a zinc finger in its regulatory domain, and of the zinc finger DNA repair enzyme Fpg. NO inhibits alcohol dehydrogenase, which contains both a catalytical and a structural zinc finger domain, and this inhibition is correlated with the release of Zn^{2+} . In addition to these *in vitro* studies, we recently found NO-mediated intracellular Zn^{2+} release in live cells. NO has also been shown to inhibit the transcription factor NF- κ B via induction and stabilization of its inhibitor I κ B α and additionally to inhibit the DNA-binding activity of NF- κ B itself. DNA binding of the transcription factor AP-1 is also inhibited by NO.

Taken together, these results strongly suggest that NO can inhibit several, intracellular enzymes and profoundly affects the cellular gene transcription machinery.

ROLES OF NO IN THE CVS

As mentioned previously, different cell types express one or more of the three isoforms of NOS; nNOS, iNOS and eNOS). nNOS is expressed in sympathetic nerve terminals and regulates the release of catecholamines in the heart. eNOS constitutively expressed in endothelial cells inhibits contractile tone and the proliferation of underlying vascular smooth muscle cells, inhibits platelet aggregation and monocyte adhesion, promotes diastolic relaxation, and decreases O_2 consumption in cardiac muscle through produced NO. eNOS is also constitutively expressed in cardiac myocytes from rodent and human species, where it opposes the inotropic action of catecholamines after muscarinic cholinergic and β -adrenergic receptor stimulation. iNOS gene transcription and protein expression are induced in all cell types after exposure to a variety of inflammatory cytokines. Aside from participating in the immune defense against intracellular microorganisms and viruses, the large amounts of NO produced mediate the vasoplegia and myocardial depression characteristic of systemic immune stimulation and promote cell death through apoptosis. In cardiac myocytes, NO may regulate L-type calcium current and contraction through activation of cGMP-dependent protein kinase and cGMP-modulated phosphodiesterases. Other mechanisms independent of cGMP elevations may operate through interaction of NO with heme proteins, non-heme iron, or free thiol residues on target signaling proteins, enzymes, or ion channels. Given the multiplicity of

NOS isoforms expressed in cardiac muscle and of the potential molecular targets for the NO produced, tight molecular regulation of NOS expression and activity at the transcriptional and posttranscriptional level appear to be needed to coordinate the many roles of NO.

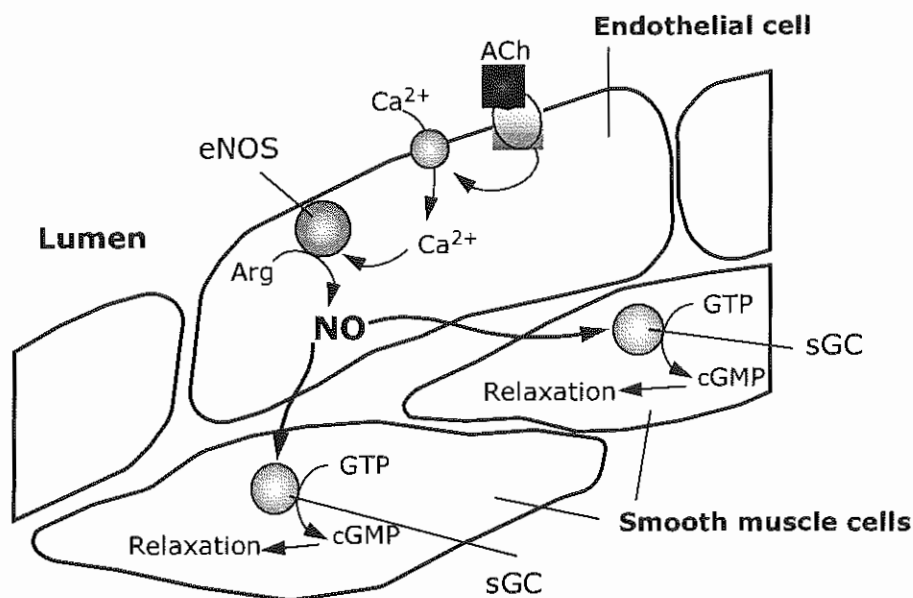


Figure 2. Signal transduction pathway involving NO and cGMP that leads to vasodilation.

Far from being only an anatomic barrier to prevent the extravasation of circulating blood into the vessel wall, the endothelium is a metabolically active organ system that maintains vascular homeostasis by (a) modulating vascular tone, (b) regulating solute transport into cell components of the vessel wall, local cellular growth, and extracellular matrix deposition, (c) protecting the vessel from the potentially injurious consequences of substances and cells circulating in blood, and (d) regulating the hemostatic, inflammatory, and reparative responses to local injury. However, a growing list of conditions, including hypercholesterolemia, systemic hypertension, smoking, diabetes, congestive heart failure, pulmonary hypertension, estrogen deficiency, hyperhomocysteinemia, and the aging process itself, have been associated with impaired functions of the endothelium. As a result, the vessel wall in these conditions may promote inflammation, oxidation of lipoproteins, smooth muscle proliferation, extracellular matrix deposition or lysis, accumulation of lipid-rich material, platelet activation, and thrombus formation. All of these consequences of endothelial dysfunction may contribute to development and clinical expression of atherosclerosis.

REGULATORY ROLE OF NO

In their classical experiment, Furchgott and Zawadzki found that strips of rabbit aorta with intact endothelium relaxed in response to acetylcholine but constricted in response to this same agonist when the endothelium had been rubbed off. The substance responsible for the acetylcholine-stimulated relaxation was initially called endothelium-derived relaxant factor, and subsequently found to include nitric oxide. It is now known that a

variety of agonists (e.g., acetylcholine, histamine, thrombin, serotonin, ADP, bradykinin, norepinephrine, substance P, and isoproterenol) can increase the synthesis and release of nitric oxide from the endothelium, although many of these same agonists (e.g., acetylcholine, serotonin, norepinephrine, and histamine) constrict vascular smooth muscle in the absence of endothelium. Vasoactive substances produced within the endothelium, such as bradykinin, may also stimulate nitric oxide release by autocrine and paracrine effects on endothelial B2 kinin receptors. However, the principal physiologic stimulus for nitric oxide synthesis and release from the endothelium is likely the shear stress of blood flowing over the surface of the vessel by a nonreceptor-dependent mechanism. Nitric oxide, released from the endothelium as a gas or attached to other molecules, stimulates soluble guanylyl cyclase, producing increased concentrations of cyclic GMP. Depending on the direction of nitric oxide release and the site of cyclic GMP activation, differing biological effects can be observed. For example, increased cyclic GMP in vascular smooth muscle cells underlying the endothelium activates GMP-dependent kinases that decrease intracellular calcium, producing relaxation, whereas increased cyclic GMP in platelets by action of nitric oxide released into the blood vessel lumen decreases platelet activation and adhesion to the surface of the endothelium. Nitric oxide also regulates the cellular environment within the vessel wall by inhibiting the activity of growth factors released from cells within the vessel wall and from platelets on the endothelial surface. Nitric oxide has antiinflammatory properties by inhibiting the synthesis and expression of cytokines and cell adhesion molecules that attract inflammatory cells to the endothelial surface and facilitate their entrance into the vessel wall. This effect of nitric oxide may be mediated by inhibition of the activation of an important nuclear transcription factor (nuclear factor κ B) that binds to the promoter regions of genes that code for proinflammatory proteins. NO also governs basal systemic, coronary, and pulmonary vascular tone by increased cGMP in smooth muscle, by inhibition of a potent constrictor peptide, endothelin-1, and by inhibition of the release of norepinephrine from sympathetic nerve terminals.

Thus, NO plays a pivotal role in regulating vessel wall homeostasis. Although the endothelium-dependent processes involve a multitude of metabolic and gene transcriptional pathways, NO either directly or indirectly plays an important role in their regulation.

Table 1. Molecular targets of NO relevant to CVS functions.

Mechanism	Target Molecule	Functional Effect
S-Nitrosation of thiol residues	GADPH (Cys ¹⁴⁹)	Reversible inhibition of active site. Irreversible inactivation through ADP ribosylation.
	NADPH oxidase	Enzyme inhibition.
	Glutathione	Depletion of intracellular stores of reduced forms.
	Tissue plasminogen activator	Activation of vasodilation and antiplatelet properties.
	Hemoglobin	Allosterically controlled exchange of NO to other acceptors.
	Ca ²⁺ -activated K ⁺ channel	Vasodilation.
Binding to non-heme iron	Aconitases	Mitochondrial: inhibition of tricarboxylic acid cycle. Cytosolic (IRE-BP): regulation of iron metabolism.
	Mitochondrial electron transport chain (complexes I, II, and IV)	Inhibition of high-energy phosphate metabolism.
	Ribonucleotide reductase	Inhibition of DNA synthesis.
Interaction with heme proteins	NOS	Inhibition of NO production.
	Cyclooxygenase 2	Activation of prostaglandin and thromboxane formation.
	Guanylyl cyclase	Activation of cGMP formation.
	Myoglobin, hemoglobin	Inactivation of NO.
Tyrosine nitration	Cytoskeletal proteins, contractile myofilaments	Contractile dysfunction?
Oxidation	DNA strand breaks	Activation of poly-ADP ribose synthase, depletion of cell energy stores, and contractile dysfunction.

ROLES OF NO IN THE CNS

Intensive research over recent years has established that NO serves as an endogenous messenger molecule in the nervous system and mediates a variety of unique functions as part of the processing of neural signals. NO is an unconventional transmitter since it is not packaged in synaptic vesicles but, rather, diffuses from its site of production and moves readily through cell membranes. In nerve cells, NO is generated in an activity-dependent process by nNOS, Ca^{2+} -calmodulin-stimulated enzymes. The principal function of NO appears to be as an activator of sGC, one of the cGMP-synthesizing enzymes. The stimulation of sGC is caused by the binding of NO to the iron-containing heme group in the active center of sGC. Despite the fact that NO diffuses in neuropilar compartments, the specificity of cellular communication is preserved by the activity-dependent release of the ligand and discrete distribution of the target receptor. Since NO is a free radical, other, cGMP-independent, signal-transduction pathways are also possible.

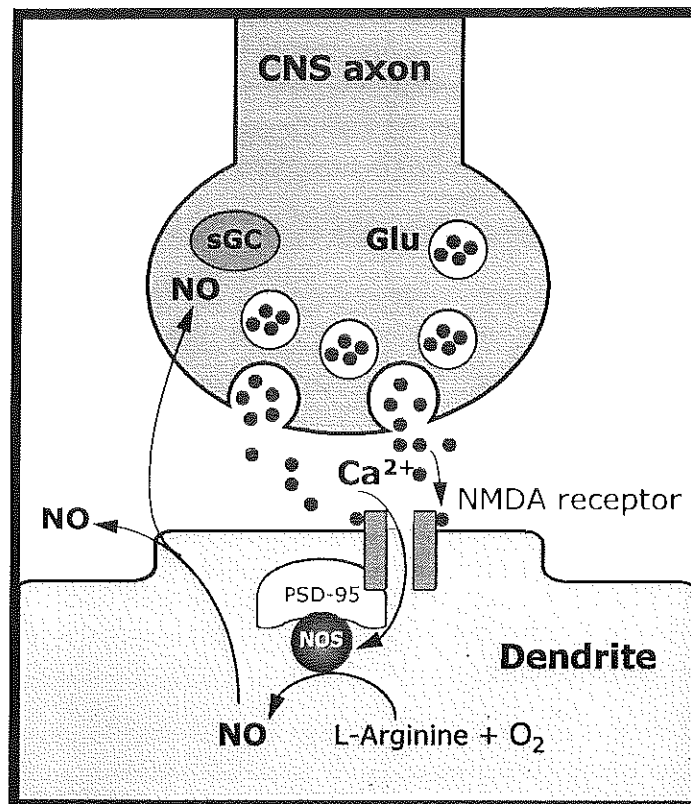


Figure 3. Central synaptic regulation of nNOS. Protein interactions with nNOS target the synthase to discrete sites in excitable cells. These interactions likely account for differential regulation of nNOS by specific calcium influx pathways. Associations with PSD-95 mediates coupling of nNOS to NMDA receptor activity in the CNS.

NO signaling requires rapid and controlled delivery of NO to specific cellular targets. Other neurotransmitters are packed in secretory vesicles that are released at synaptic sites. Signal termination is mediated by enzymes and pumps that eliminate the active transmitter from the synapse. Regulation of NO signaling is complicated by the physical properties of NO, which prevent storage of NO in lipid-lined vesicles or metabolism of NO by hydrolytic enzymes. In addition, excessive production of NO is toxic to neurons

and other cells. Therefore, NO signaling must allow for rapid and localized NO production and immediate termination of biosynthesis. This tight control of NO signaling is largely regulated at the level of NO biosynthesis. Indeed, the NOS proteins are among the most highly regulated of all neuronal enzymes. Acute control of nNOS activity is mediated by allosteric regulation, by posttranslational modification, and by subcellular targeting of the enzyme. nNOS protein levels are also dynamically regulated by changes in gene transcription, and this affords long-lasting changes in tissue NO levels.

nNOS activity is primarily regulated by local increases in intracellular calcium, which stimulates nNOS through interaction with calmodulin. Distinct calcium influx pathways specifically regulate nNOS in various tissues. In the myenteric nervous system where NO functions as a neurotransmitter, NOS activity is primarily regulated by calcium influx through voltage-dependent calcium channels. Intestinal relaxation mediated by NO is suppressed by the N-type calcium channel antagonist, ω -conotoxin. In the brain, NO biosynthesis is predominately regulated by calcium influx at the synapse. Glutamate, the major excitatory neurotransmitter in the brain, is the most effective activator of NO biosynthesis in most brain regions. Calcium influx through the N-methyl-D-aspartate (NMDA) receptor potently activates nNOS in brain. NMDA receptors are also known to play a critical role in learning and memory; the intimate relationship of NMDA receptors with nNOS helps to explain the role of NO in memory consolidation.

NO IN LEARNING AND MEMORY FORMATION

In the CNS, NO acts as a diffusible intercellular signaling molecule. A role for NO has also been suggested in the mechanisms of synaptic plasticity, including long-term potentiation (LTP) in the hippocampus, long-term depression (LTD) in the cerebellum, learning and memory formation *in vivo*, and the expression of behavioral sensitization and tolerance to psychostimulants. In LTP and LTD, cellular models of memory, repeated neuronal stimulation yields long-lasting changes in synaptic strength. NOS inhibitors prevent these changes. Studies with NOS inhibitors have been controversial because these arginine analogues often have nonspecific effects. This controversy may now be resolved by studies of NOS knockout mice. Both eNOS and nNOS activities are found in hippocampus. Mice that lack either eNOS or nNOS have essentially normal LTP, whereas mutant mice deficient in both eNOS and nNOS have substantially decreased LTP.

Memory impairment in aged rats may be accompanied, at least in part, by a decrease in NO production in the brain. More recently, a specific nNOS inhibitor, 7-NI impairs spatial reference memory formation in the radial arm maze task while L-NAME, a non-selective NOS inhibitor, inhibits both reference and working memories. Furthermore, it has been demonstrated that an NO precursor, L-Arg, increased the choice accuracy, by reducing reference memory errors, of the radial arm maze task in the late phase of training. These results suggest that NO plays a significant role in spatial memory formation, especially in reference memory.

NEUROPATHOLOGICAL ROLE OF NO

Studies have demonstrated that NO is involved in NMDA receptor-mediated neurotoxicity and convulsions, and in the neuronal death that occurs after focal cerebral ischemia. Under certain circumstances NO synthesis may be excessive and NO may become neurotoxic. Excessive glutamate-receptor stimulation may lead to neuronal death through a mechanism implicating synthesis of both NO and superoxide ($O_2^{\cdot -}$) and hence

peroxynitrite (ONOO^-) formation. In response to lipopolysaccharide and cytokines, glial cells may also be induced to synthesize large amounts of NO, which may be deleterious to the neighbouring neurones and oligodendrocytes. The precise mechanism of NO neurotoxicity is not fully understood. One possibility is that it may involve neuronal energy deficiency. This may occur by ONOO^- interfering with 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. Possible mechanisms whereby ONOO^- impairs the mitochondrial respiratory chain and the relevance for neurotoxicity are discussed. The intracellular content of reduced glutathione also appears important in determining the sensitivity of cells to ONOO^- production. It is concluded that neurotoxicity elicited by excessive NO production may be mediated by mitochondrial dysfunction leading to an energy deficiency state.

NO AND MITOCHONDRIAL FUNCTIONS

NO has been shown to change ion currents through the mitochondrial membrane leading to release of Ca^{2+} into the cytosol. It is long known that activated macrophages inhibit the mitochondrial respiration of target cells. In the mitochondrial membrane, ATP is produced through the coupling and successive reduction of NADH to NAD^+ by oxidizing O_2 to H_2O via a gradient of enzyme redox potentials within the electron transport chain. The enzymes directly involved are complexes I, III, IV, and V (today complex II is known not to be part of this cascade but to supply electrons from the citric acid cycle). Although there are various hemes and Fe-S clusters present (more exactly, hidden) in any of these protein complexes, only the cytochrome c oxidase (complex IV) is inhibited by NO via binding to its heme moiety in a reversible manner. This is analogous to the well-studied inhibitory effects of CO and CN^- , which are isoelectronic to NO.

Complexes I and III are relatively insensitive to NO. However, $\text{O}_2^{\cdot -}$ is a by-product of the mitochondrial respiratory electron transport and its production is enhanced in the presence of electron transport chain inhibitors such as rotenone or antimycin A or CN^- . By analogy, (reversible) inhibition of complex IV by NO may cause a (transient) inhibition of the electron flow yielding increased $\text{O}_2^{\cdot -}$ synthesis by complexes I and III. $\text{O}_2^{\cdot -}$ and NO may then react to the strong oxidant peroxynitrite anion (ONOO^-) which has been shown to irreversibly inhibit complexes I, II, and III but not complex IV. In conclusion, the current hypothesis is that a reversible NO-mediated inhibition of the respiratory chain may result in enhanced intracellular $\text{O}_2^{\cdot -}$ and subsequent ONOO^- production. This may lead to peroxidation of lipids, to destruction of Fe-S clusters within enzymes, and to inhibition of the mitochondrial manganese superoxide dismutase via nitration and may furthermore induce Ca^{2+} efflux from the mitochondria. This altogether causes irreversible damage to the power stations of the cell.

NO AND THE NUCLEUS

The nucleus is a further cellular target for NO. NO has been shown to cause G:C - A:T transitions and to mediate DNA strand breaks, both suggested to be the results of N-nitrosylation of deoxynucleotides, thus yielding deaminated DNA bases. However, indirect induction of DNA strand breaks, e.g., via intracellular oxygen radical and/or peroxynitrite anion generation, via N-nitrosamine formation and subsequent alkylation reactions, via activation or inhibition of enzymes necessary for nuclear homeostasis, or

via other mechanisms, cannot be excluded to date. Indeed, NO has been shown to induce oxidative DNA damage in an activated macrophage cell line and to inhibit enzymes involved in DNA repair. Because DNA damage is a constant hazard in natural environments induced by chemicals, ionizing radiation, or UV light, leading to a variety of biological consequences such as mutation induction, blocking of transcription, and replication, cells have evolved an array of mechanisms for repair. Recent observations suggest that damaged DNA is processed not only by DNA repair enzymes but also by other nuclear factors involved in a variety of cellular functions. Most forms of DNA alterations are recognized by DNA excision repair pathways catalyzing removal of damaged or modified regions. Thus, strand breaks induced by endonucleases at active repair sites serve to signal the presence of DNA damage, which is then repaired by polymerization and ligation. Proliferating cells are especially vulnerable to DNA damage due to the added demands of cellular growth and division. Delaying progression through the cell cycle at so called "cell-cycle checkpoints" provides the time necessary for repair. In addition to cell-cycle arrest, DNA damage can induce apoptosis (programmed cell death) in cells of multicellular organisms, thus eliminating cells in which damage is beyond repair possibilities, thereby preventing propagation of mutations. Initiation of either cell-cycle arrest or apoptosis requires induction of the tumor-suppressor protein p53, the main biological function of which is to be a "guardian of the genome". Another important protein involved in DNA repair is the poly(ADP-ribose)polymerase (PARP). The PARP is a constitutively expressed nuclear protein (approximately 10^6 copies/nucleus) which is regarded as a molecular nick sensor and has a functional role during rejoining of DNA strand breaks. Following its binding to DNA breaks, PARP automodifies itself by adding several branched polymer chains of up to 200 ADP-ribose residues each resulting in PARP inhibition and causing its dissociation from the DNA strand breaks. The poly(ADP-ribose)polymers synthesized in response to DNA damage are then degraded within 1-2 min by specific glycohydrolases. The physiological role of the PARP is not exactly known to date. It either protects DNA strand breaks during early stages of recombination and repair or it may transiently block DNA replication, thus inducing a cell-cycle arrest and providing time or space for assembly of the DNA repair complex. It may also simply constitute an emergency signal. Whatsoever the exact roles of p53 and PARP are, induction of p53 protein expression and activation of PARP serve as an indirect indicator for DNA damage. NO treatment has been shown to induce p53 expression and to activate PARP in neurons and in islet cells. While p53 expression per se is not detrimental for cells, activation and subsequent poly(ADP-ribosylation) of PARP lead to a severe cellular depletion of ATP and NAD^+ . Consequently, PARP inhibitors have been shown to partially protect islet cells and neurons from NO-mediated cell death and to inhibit NO-mediated cellular NAD^+ depletion in islet cells. In addition, NO treatment of islet cells isolated from mice with a disrupted PARP gene did not result in NAD^+ depletion and, hence, these cells exhibited an increased resistance toward NO.

In conclusion, NO mediates DNA damage, thereby causing depletion of cellular ATP and NAD^+ levels which may contribute to cell lysis.

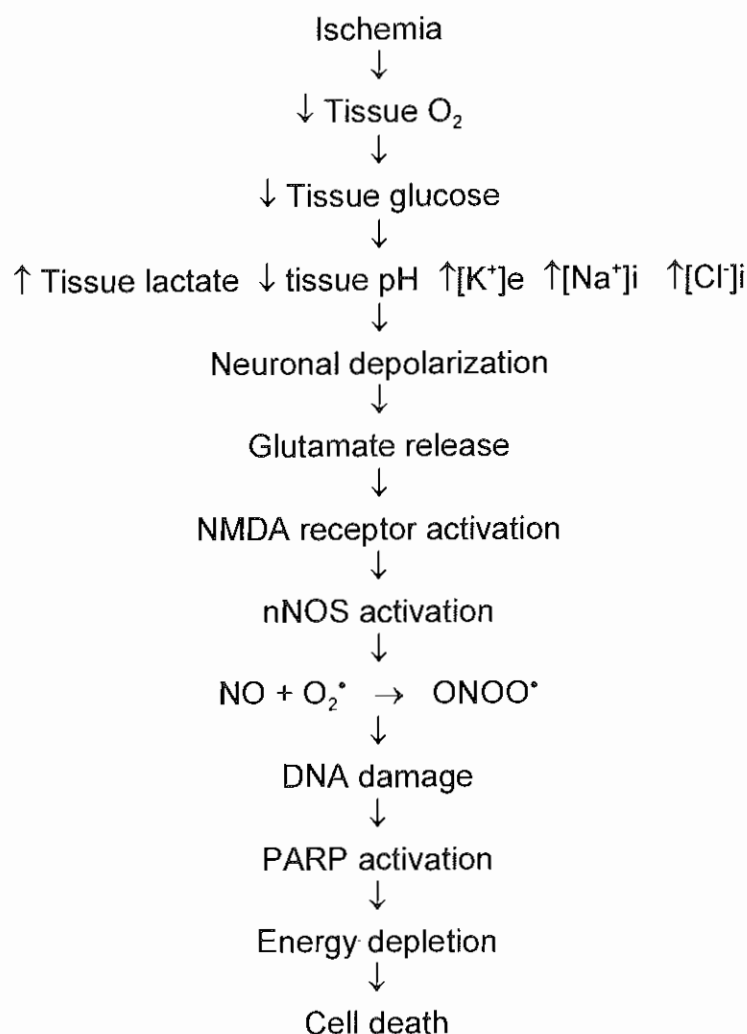


Figure 4. A model for the role of PARP in neurotoxicity due to ischemia. Following ischemia, compromised neuronal metabolism leads to a more positive resting potential and subsequent depolarization and neurotransmitter release. Excessive neurotransmitter release leads to increased production of NO which combines with superoxide to form peroxynitrite. Peroxynitrite may lead to DNA strand breaks and subsequent activation of PARP. Massive activation of PARP depletes NAD and consequently ATP.

NO IN NEURODEGENERATIVE DISEASES

Although NO clearly participates in neuronal injury after vascular stroke and cerebral ischemia, the role of NO in human neurodegenerative disease is not as easily understood. The slow progression of these diseases, occurring over 50 years or more, complicates experimental approaches to modeling their pathophysiological mechanism. However, histopathological evidence suggests that certain neurodegenerative diseases may also be mediated by NO and glutamate toxicities. For example, NADPH-diaphorase positive neurons in the corpus striatum, which are the NOS neurons, are selectively spared in Huntington's disease. This selective pathology can be replicated in striatal culture models and *in vivo* after lesions with NMDA but not other classes of glutamate agonists. The

spared NADPH diaphorase neurons are uniquely endowed with high levels of superoxide dismutase (SOD), which may protect the cells from peroxynitrite-mediated NO neurotoxicity. Indeed, 3-nitrotyrosine, the footprint of peroxynitrite, is detected in striatal neurons in animals models of Huntington's disease.

Although stigmata of NO toxicity only correlate with Huntington's disease pathology, a more causal role for NO and peroxynitrite toxicity has been established in some forms of Parkinson's disease. MPTP, which contaminated batches of illicit drugs in the 1970s, produces Parkinsonian-like symptoms in humans. MPTP causes pathology by targeting the destruction of nigrostriatal dopaminergic neurons, the same cells that are selectively lost in idiopathic Parkinson's disease. Treatment of experimental animals, including mice and primates, with MPTP replicates this selective toxicity and results in accumulation of 3-nitrotyrosine in the nigrostriatal pathway. Inhibition of NOS prevents both the neurotoxicity of MPTP and the associated formation of 3-nitrotyrosine. Definitive evidence that NO and peroxynitrite mediate toxicity in the MPTP model of Parkinson's disease again derives from studies of transgenic mice. Both nNOS knockout mice and mice that overexpress Cu/Zn SOD are resistant to MPTP toxicity.

Although nNOS inhibitors can prevent acute toxicity associated with MPTP, it remains less clear whether long-term treatment would be therapeutic for slowly developing neurodegenerative disorders. Chronic animal models for these diseases will first need to be established, and then the role of NO can be evaluated rigorously. The recent identification of a Parkinson's disease gene, and the development of transgenic animal model for Huntington's disease, suggests it will not be long until we NO (know).

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