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Perspective

Nitric Oxide: A New Paradigm for Second Messengers

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Introduction

NO, more than any other biological second messenger discovered to date, manifests a new paradigm for the way a molecule can regulate the biological environment in which it is produced. Its physiological effects are attributed to a very small, freely diffusible, and potentially highly reactive radical. NO regulation, signal transduction, and cytotoxicity is strictly dependent upon its chemical reactivity with oxygen and metals rather than specific structural interactions with physiological targets.[1]

It is difficult to conceive that a molecule that is now known to have numerous important functions in mammalian tissues and a structure as simple as nitrogen–oxygen would elude detection until the last decade. In part, the failure to recognize NO as an important and ubiquitous endogenous second messenger or cytostatic/cytotoxic agent was a result of its high chemical reactivity in biological systems and, thus, very short half-life (5–10 s in vitro).[2] In recent years the development of techniques to analyze for NO and its reaction products in biological media coupled with the availability of specific inhibitors of the enzymes that synthesize NO has dramatically increased the understanding of the role of NO in mammals. Since 1980 evidence demonstrating that NO is an important second messenger which is involved in the homeostatic regulation of blood pressure, blood clotting, and neurotransmission has accumulated. Furthermore, NO has been shown to serve as part of the host defense system against cancerous cells and intracellular parasites and microbes.

Physical Properties of NO

NO in the pure state under standard temperature and pressure is a gas. With the exception of the lung (e.g., in the presence of a gaseous phase), NO acts as a dissolved nonelectrolyte in all its biological activities. Thus, under virtually all biologically relevant conditions, NO is not a "gas".

NO is moderately soluble in water, with a concentration in a saturated solution (1 atm of headspace NO) of 1.9 mM (25 °C).[3] NO is much more soluble in apolar solvents such as *n*-hexane, with a concentration at saturation of 0.13 M;[3] thus NO will tend to dissolve selectively in the membrane and lipid phases of cells. Indeed, measurements using NO-selective microsensors have demonstrated a higher concentration of NO immediately adjacent to the plasma membrane of an NO-producing endothelial cell.[4] This results in an increase in the bidirectional diffusion of NO and the establishment of a "reservoir" of NO within membrane structures. However, it is important to note that the total volume of the aqueous phase is much greater than the volume within the bilayer phase of cell membranes. The diffusion constant (D) of NO in water is in the general range $(2-4) \times 10^{-5}$ cm^2/s and significantly increases with temperature.[5] Using microsensors under physiologically relevant conditions (including 37 °C), D can be calculated as 3.3×10^{-5} cm^2/s. Using this value of

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D, simulations of the diffusion of NO reveal that this molecule travels surprisingly large distances from the cell that produces it before inactivation.[6]

Chemical Properties of NO

NO is an uncharged molecule. Because of its 11 valence electrons, it contains one unpaired electron and is paramagnetic, a property which dominates its chemistry. The most common chemical interactions of NO in biological systems are characterized as stabilization of the unpaired electron. In general, this occurs either through the reaction of NO with another paramagnetic species (e.g., O_2, $O_2^{\bullet -}$, or peroxy radicals) or by complexation of NO to a metal. In the former case, the eventual result is the formation of a stabilized diamagnetic species. In the latter case, the unpaired electron is shared by both NO and the metal, and the stability is imparted by delocalization of the unpaired electron in the d orbitals of the metal. Both of these general reactions are described in further detail below.

Reaction of NO with Oxygen Species

The gas-phase reaction of NO with O_2 has been studied for many years, primarily because of its importance in atmospheric pollution. In this reaction, two molecules of NO react with one molecule of O_2 to produce two molecules of another paramagnetic radical, nitrogen dioxide (NO_2):

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

In aqueous solution, a similar reactant stoichiometry holds, i.e., the reaction is second order in NO and first order in O_2.[7] In the gas phase, NO_2 will react further either with another NO_2 to form dinitrogen tetroxide (N_2O_4) or with another NO to form dinitrogen trioxide (N_2O_3):

$$NO_2 + NO_2 \rightarrow N_2O_4$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (2)$$

These species, as well as nitrous acid (HNO_2), are highly reactive, and in aqueous solution they may be considered donors of nitrosonium ion [NO^+].

The chemistry of the transfer of the nitrosonium group is known as transnitrosation and is undoubtedly the most studied reaction of nitrogen oxides.[8] This chemistry is characterized by nitrosonium transfer to a variety of nucleophiles, including halides, nitrogen, and sulfur. In water, transfer to hydroxyl yields nitrous acid, and so dissolution of N_2O_3 yields nitrite and dissolution of N_2O_4 yields nitrite and nitrate.

$$[NO^-] + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^-$$

$$[NO^-][NO_2^-] + H_2O \rightarrow 2NO_2^- + 2H^-$$

$$[NO^+][NO_3^-] + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (3)$$

The sole isolated product of the reaction of NO with O_2 in water is nitrite,[7] which may indicate the intermediary formation of N_2O_3, although this is not completely clear.

Two other biologically important nucleophilic targets for nitrosation are nitrogen and sulfur. In the case of nitrogen, the product is a nitrosamine.

$$R_2NH + [NO^+] \rightarrow R_2NNO + H^- \quad (4)$$

In the case of secondary amines, the nitrosamine is stable, but such molecules are potent mutagens and carcinogens[9] and their formation from enzymatically generated nitrogen oxides has been implicated in the etiology of a variety of cancerous states predisposed during inflammation.[10]

Sulfur is also a target of nitrosation,[11] and in the case of thiols, an S-nitrosothiol (SNT) is formed.

$$RSH + [NO^-] \rightarrow RSNO + H^+ \quad (5)$$

For as yet unknown reasons, the stabilities of SNTs are very dependent on the nature of R. Two exceptionally stable SNTs are S-nitroso-N-acetylpenicillamine (SNAP) and the SNT derivative of glutathione (GSNO). SNTs undergo three major reactions: homolytic scission involves the reaction of the S–N bond and separation of the bonding electrons to form NO and a thiyl radical (two thiyl radicals combine to form the disulfide); heterolytic scission involves the reduction of the SNT to the thiol and NO; and SNTs also can act as nitrosonium donors. Thus the rate of formation of NO from SNTs is affected by a variety of agents, including oxygen, superoxide, metals, other thiols, and also cellular metabolism. NO will not react directly with thiols to form SNTs, nor will it react with amines to yield nitrosamines. However, if the reaction is exposed to molecular oxygen (O_2) the nitrosation will occur.

NO reacts extremely rapidly with superoxide to produce initially the reactive species peroxynitrite.

$$NO + O_2^{\bullet -} \rightarrow ONOO^- \quad (6)$$

Peroxynitrite is a potent oxidant, capable of oxidizing thiols[12] and DNA bases[13] and initiating metal-independent lipid peroxidation.[14] In the presence of certain metals, e.g., iron, peroxynitrite undergoes heterolytic cleavage to form a nitronium-like (NO_2^+) species which can nitrate phenolic compounds, including the aromatic ring of tyrosine in proteins.[15,16]

Under physiological conditions the conjugate acid of peroxynitrite, peroxynitrous acid (ONOOH, $pK_a = 6.8$ at 25 °C),[17] is highly reactive and possesses the reactivity of hydroxyl radical. Homolytic cleavage of ONOOH will generate the reactive radical $^{\bullet}NO_2$, which may also contribute to peroxynitrous acid toxicity.[18] However, a certain and probably variable proportion of the ONOOH spontaneously rearranges and deprotonates to form the unreactive nitrate anion.[19]

Based solely on the chemistry of the reaction of NO with $O_2^{\bullet -}$, it is clear that production of peroxynitrite and peroxynitrous acid will be damaging to biomolecules. Alternatively, it is also possible that the formation of nitrate will result in mutual scavenging of NO and $O_2^{\bullet -}$.

NO will also react with organic peroxyl radicals.[20] This reaction may be an antioxidant activity of NO, whereby lipid radical chain reactions are terminated by NO reacting with lipid alkoxyl and peroxyl radicals. Products of this reaction include nitrito, nitro, nitrosoperoxo, and nitrated lipids. Another potential antoxidant activity of NO may be its reaction with peroxynitrite.[20]

Reaction of NO with Metals

Because the partially filled d orbitals of many biologically important metals lie relatively close together in

energy, they are excellent homes for unpaired electrons such as that in the NO molecule. In addition, many proteins such as hemoglobin, myoglobin, cytochrome oxidase, peroxidases, dioxygenases, and the cytochromes P_{450} bind dioxygen as an integral part of their function, and so the close structural similarity renders them targets of NO. Metal–nitrosyl complexes[21] have been known for nearly 150 years, but until relatively recently it was generally assumed that, with a few well-known exceptions such as the nitroprusside ion, these complexes were not very reactive. However, it is now known that the bonding characteristics of metal nitrosyls can dictate great changes in the reactivity of the coordinated nitrosyl group. Under biological conditions, metal–nitrosyl complexes can liberate NO or nitroxyl anion (NO^-) or can act as effective nitrosating agents through (NO^+) donation.

NO has been used as a dioxygen analogue for nearly 130 years,[21] and a great deal of information is available on its interaction with a variety of metalloproteins. NO is used as a substitute for O_2 since the nitrosyl complexes of many metal-containing centers are paramagnetic, and so electron paramagnetic resonance (EPR) spectroscopy can be used to yield a great deal of information about the metal center environment. EPR signals are very sensitive to the identity and oxidation state of the metal as well as to the nature and spatial arrangements of the multiple ligands around the metal center. The EPR spectra of NO complexes with metalloproteins that contain heme iron or nonheme iron as well as copper have been reported.[22]

NO exposure results in the reversible and/or irreversible inhibition of a number of heme- and nonheme-iron-containing enzymes, including nitrogenase, hydrogenase, cytochrome oxidase, cytochrome P_{450}, mitochondrial electron transfer, pyruvate–ferredoxin oxidoreductase, chloroplast photosystem II, cytochrome aa_3 of *Pseudomonas denitrificans*, nitrous oxide reductase, cytochrome cd_1 from *Pseudomonas aeruginosa*, several nitrite reductases, and nitric oxide synthase (NOS).[23] However, inhibition by addition of reagent quantities of NO in the test tube does not necessarily mean that this effect is biologically important under either physiological or pathophysiological conditions. In mammalian cells the half-life for NO is relatively short (5–10 s)[2] (and so the maximum concentrations are in the micromolar range) and the formation of higher nitrogen oxides from the reaction of NO with O_2 may well be more damaging to cellular targets than NO itself.

Interplay between Reactions of NO in Biological Systems

When NO is produced by cells in vivo as well as in vitro, it is highly likely that the predominant biological effects of NO will be dictated by the precise balance between a variety of different, simultaneously occurring, reactions. The cellular consequences of NO formation will be dictated by (1) which types of nitrogen oxide species are formed, (2) their relative rates of formation, (3) the potential targets available in the vicinity, and (4) the rapidity with which they react with these species. The interplay between different possible reaction pathways of NO can yield remarkable complexity in the patterns of its actions in biological systems, underlying its importance.

Quantitatively, the most important reaction of NO in mammals is its reaction with oxyferrohemoglobin. This reaction is a transfer of O_2 plus an electron to NO, forming nitrate anion and oxidized (met) hemoglobin:

$$Hb(Fe^{2+})O_2 + NO \rightarrow Hb(Fe^{3+}) + NO_3^- \qquad (7)$$

This reaction is extremely fast,[24] and coupled with the very high heme concentration (ca. 25 mM) in the bloodstream, this means that in vivo NO synthesis can result in substantial amounts of circulating NO_3^-. NO also reacts with deoxyferrohemoglobin to form the nitrosyl complex, and this reaction is also extremely rapid.[25] In an aerobic environment, once formed, the nitrosylhemoglobin complex will react with O_2 (also yielding nitrate), but the reaction has not been examined in detail.

The half-life for the reaction of NO with O_2 in in vitro biological systems (5–10 s[2,4]) is much faster than that predicted from the reaction kinetics measured in water.[26] However, even though the mechanisms for the oxidation of NO in biological systems are not clearly understood, at least one highly reactive species formed from NO oxidation is capable of nitrosation,[27] which may well have relevance in the etiology of cancer under conditions of chronic inflammation.[10]

NO Converges Biological Disciplines

The emergence of NO as a physiological mediator has resulted in the convergence of several disparate lines of biological research including the areas of cardiovascular pharmacology, immunology, and neurobiology.[28,29] In the area of cardiovascular pharmacology, it was established that the substance released from stimulated endothelial cells that causes smooth muscle relaxation, initially termed endothelium derived relaxing factor (EDRF), is actually NO.[30] Interestingly, before it was known that EDRF existed, it had been well established that NO and NO-releasing agents could cause vascular smooth muscle relaxation and inhibit platelet aggregation via activation of the enzyme soluble guanylate cyclase (sGC) that catalyzes the synthesis of c-GMP. These studies with NO and NO-releasing substances laid the foundation for determining the identity of EDRF and the mechanism(s) by which it mediates its vascular effects.

In the area of immunology, nitrogen oxides are quantitatively significant mammalian metabolic products which accompany an inflammatory response.[31] Rodent macrophages, stimulated with the inflammatory mediator lipopolysaccharide (LPS), are an important source of the nitrogen oxides[32] that are produced in amounts sufficient to inhibit DNA synthesis and the metabolism of cancer cells by interfering with essential iron–sulfur-containing mitochondrial enzymes.[33] The cytostatic effect as well as the production of nitrogen oxides by macrophages is L-arginine (Arg) dependent,[33] leading to the hypothesis that the macrophage-derived reactive nitrogen oxide product is NO and NO has been shown to mimic the cytostatic effects observed with physiological NO.[34]

The second messenger role for NO in the central and peripheral nervous systems followed from studies in the cardiovascular and immune systems. Rat cerebellar slices were found to release a labile substance upon activation of the *N*-methyl-D-asparate (NMDA) receptor which stimulated sGC.[35] This labile substance had properties similar to EDRF, and cGMP synthesis was inhibited by L-N^G-methylarginine (NMA, **1**), a compound

Table 1. Comparison of NOS Isoforms

parameter	human NOS-I[38]	human NOS-II[39]	human NOS-III[40]
chromosomal localization	12q24.2	17cen-q12	7q35-36
amino acid length	1433	1153	1203
calculated M_r (kDa)	160	130	133
native structure	dimer	dimer	undetermined
Swiss potein no.	P29475	P35228	P29474
apparent K_m (L-Arg, μM)	2	2.5	0.9 ± 0.04
apparent IC_{50} (μM)[41]			
NMA	3.3	5.2	1.8
NNA	0.20	3.6	0.31
NIO	3.7	1.3	3.8
Et-ITU	0.31	0.03	0.19

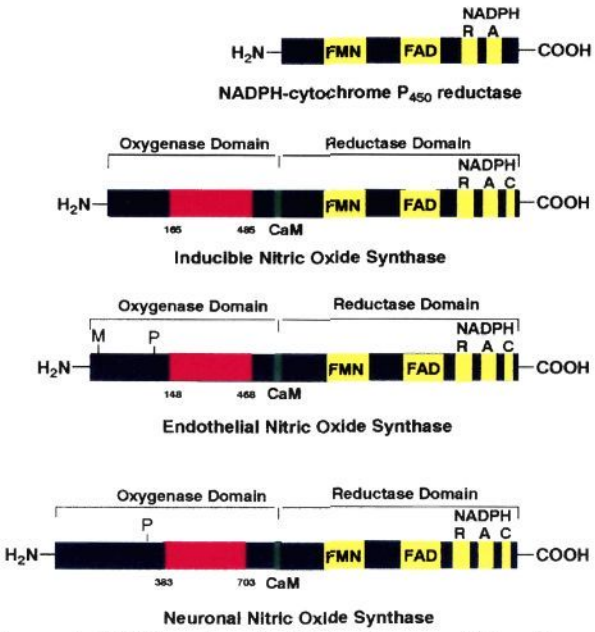


Figure 1. NOS domains. An illustrative map of the primary amino acid sequence of human NOS isozymes and NADPH cytochrome P_{450} reductase. Oxygenase and reductase domains are separated by the Ca^{2+}/calmodulin binding region (CaM, green). The reductase domain is homologous with the mammalian enzyme NADPH cytochrome P_{450} reductase. Both contain binding sites (yellow) for flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) as well as several consensus sites for the electron donor species nicotinamide–adenine dinucleotide phosphate (NADPH) (ribose moiety; NADPH-R; adenine moiety, NADPH-A; and C-terminal binding domain,[42] NADPH-C). The oxygenase domain in NOS-I and NOS-III contains a phosphorylation site (P). All NOS contain a region of ~320 residues (red) that is thought to contain the binding sites for heme, tetrahydrobiopterin, and Arg. The amino acid sequence of this region is highly conserved among the various forms of NOS.

that had previously been shown to inhibit macrophage-derived NO production.[33]

Nitric Oxide Synthase Isozymes

There are three distinct mammalian enzymes, the nitric oxide synthases (NOS, Table 1), which synthesize NO.[36,37] All three NOS isozymes have been purified, characterized, and cloned. All NOS isozymes must have calmodulin bound in order to be active. The NOS isozymes (EC 1.14.13.39) are endothelial NOS (eNOS, ecNOS, or NOS-III), neuronal NOS (nNOS, ncNOS, bNOS, or NOS-I), and inducible NOS (iNOS, mNOS, NOS-II) (see Figure 1). Both NOS-I and NOS-III are constiutively expressed whereas NOS-II requires cytokine induction in order to be expressed. NOS isozymes have been purified or cloned from several sources including rat brain, murine macrophages, rat hepatocytes, rat vascular smooth muscle cells, bovine aortic endothelial cells, human brain cDNA, human umbilical vein endothelial cells (HUVEC), human placenta, human chondrocytes, human glioblastoma cells, human hepatocytes, and a human adenocarcinoma cell line among others.[29,37]

The endothelial isozyme (NOS-III) has been cloned from human and bovine aortic endothelial cells.[37] It is localized in the particulate fraction of cells. NOS-III is myristolated[43] and palmytolated, which is a probable explanation for its localization in cell membranes. Furthermore, NOS-III is known to be phosphorylated upon stimulation of endothelial cells by shear stress, inflammatory mediators, bradykinin, and other vasorelaxant agonists. [^{35}S]-labeling studies demonstrated that phosphorylated NOS-III is translocated from the membrane to the cytosol upon agonist stimulation, and this phosphorylation of NOS-III has been proposed to regulate NOS-III activity.[44] NOS-III is expressed in the vascular endothelium which is consistent with the role of NOS in the production of EDRF.

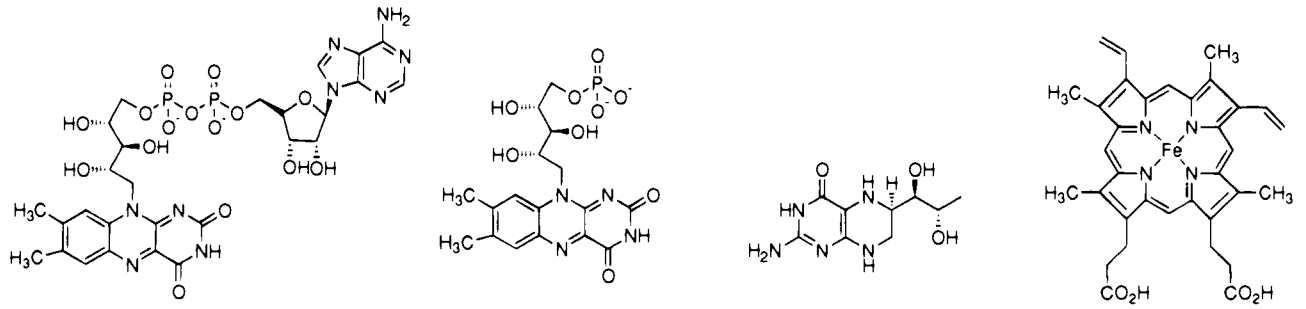
The isoform of NOS in central and peripheral neurons (NOS-I) has been cloned from rat and human cerebellar cDNA.[37] Although NOS-I is commonly referred to as the brain or neuronal isozyme, it is also formed in other tissues such as skeletal muscle, pancreatic islet cells, kidney macula densa cells, and certain epithelial cells.[45] NOS-I activity in the brain is associated with NO-mediated synaptic plasticity, whereas in the peripheral nervous system, NOS-I expression and activity is associated with smooth muscle relaxation.[46]

NOS-II is unique among the NOS isoforms in that its expression is induced by various inflammatory stimuli and its activity is independent of intracellular Ca^{2+} levels.[29] Like NOS-I and -III, NOS-II binds calmodulin, but its affinity for this complex is sufficiently great at low levels of intracellular Ca^{2+} that its activity is not regulated by normal Ca^{2+} fluxing.[47] Therefore, once induced and expressed, NOS-II continuously synthesizes NO. The expression of NOS-II is potentially ubiquitous. Upon exposure of inflammatory cytokines or LPS, NOS-II is expressed in macrophages (rodent), liver, vascular endothelial and smooth muscle cells, chondrocytes, myocardium, and other tissue and cell types.[45]

Mechanisms of NOS-Mediated NO Biosynthesis

All three NOS isozymes catalyze a five-electron oxidation of Arg to NO and L-citrulline (Cit) using reduced nicotinamide–adenine–dinucleotide phosphate (NADPH) as the source of electrons and the cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and iron protoporphorin IX (heme).[29,45,48–50] (See Figure 2 for the structures of the NOS cofactors.) Several investigators have shown that Arg is the precursor of mammalian-derived NO. Using murine NOS-II and [^{15}N]guanidino-labeled Arg, it was shown that the enzyme oxidizes one of the two equivalent guanidino nitrogens.[27] Also, elegant studies using murine NOS-II and $^{18}O_2$ and $H_2{}^{18}O$ were reported and demonstrated that the urea oxygen of the coproduct, Cit, is derived from dioxygen and not from water.[51] These experiments were extremely important because it had been hypothesized by several investigators that NOS uses water to incorporate the ureido oxygen into Cit rather than dioxygen.[33,34]

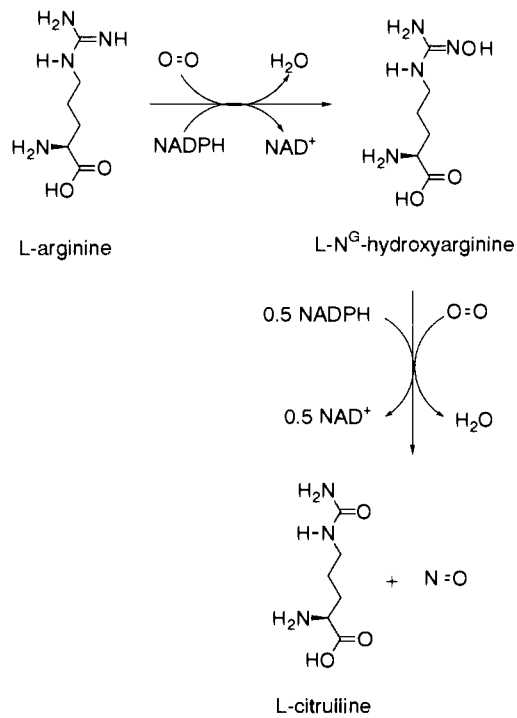
In 1988 it was first hypothesized that L-N^G-hydroxyarginine (NOHArg) was an intermediate in NO



Flavin adenine dinucleotide (FAD) Flavin mononucleotide (FMN) Tetrahydrobiopterin (BH_4) Iron protoporphyrin IX (heme)

Figure 2. NOS prosthetic groups.

biosynthesis, but no supportive data for this was reported.[34] In 1991 the synthesis of authentic NOHArg and its [^{15}N]guanidino analogs were described,[52] and these compounds were subsequently used to demonstrate that NOHArg is an intermediate in murine NOS-II-mediated NO biosynthesis.[53] In these same studies the oxidation of Arg to NOHArg was shown to require the oxidation of one NADPH and the conversion of NOHArg to NO and Cit to require an additional 0.5 NADPH. Furthermore, it was shown that the hydroxylated nitrogen of NOHArg is the same nitrogen that comprises part of the product, NO. The oxygen of NOHArg is retained in NO.[54] Recently, stoichiometry measurements have substantiated the hypothesis that two dioxygens are consumed per Arg to NO cycle.[55] Although most of these data were derived using the murine NOS-II isozyme, other studies with the NOS-I and -III isozymes are consistent with the biosynthetic pathways being conserved throughout NOS isozymes.[56] A general scheme for the biosynthesis of NO from Arg is summarized in eq 8.

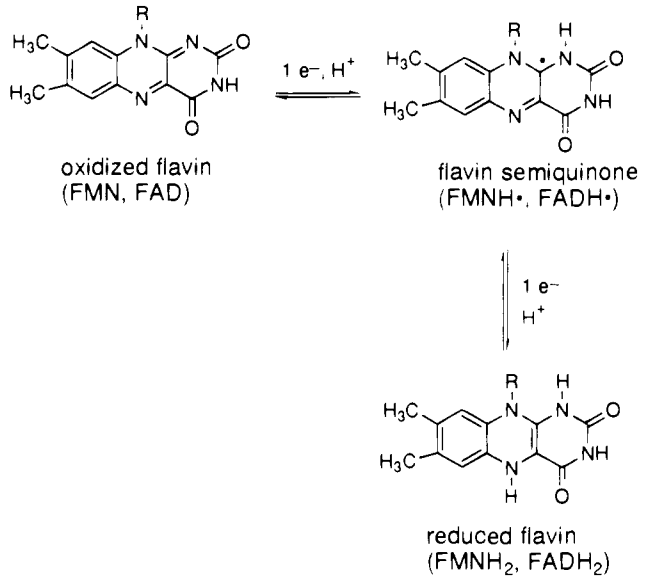


All of the studies described above, except for the oxygen stoichiometry, were done prior to the cloning of any isozyme of NOS. The subsequent cloning of NOS-I with the attendant knowledge of the amino acid sequence has allowed further elucidation of the mechanism by which NOS is able to convert Arg to NOHArg and subsequently to NO and Cit.[57] The NOS-I protein is divided into reductase and oxygenase domains (Figure 1) with the central portion of each protein containing a consensus sequence for Ca^{2+}/calmodulin binding. The C-terminal domain shares sequence homology with the mammalian enzyme, NADPH cytochrome P_{450} reductase.[57] This domain, which binds NADPH, FAD, and FMN, serves as the source (NADPH) and repository (FAD, FMN) for electrons which are transferred to the heme in the oxygenase domain of NOS. Although the oxygenase domain of NOS does not share homology with any other mammalian protein, among the NOS isozymes there is a highly conserved 320 amino acid region which may represent the binding sites for BH_4, Arg, and heme (Figure 1). On the basis of studies with NOS and other cytochrome P_{450} enzymes, the probable flow of electrons is as depicted in eq 9 with the activation of O_2 and subsequent oxidation of Arg and NOHArg to NO occurring at the heme site.[50,56]

$$\text{NADPH} \rightarrow [\text{FAD} \rightarrow \text{FMN}] \rightarrow \text{heme} \qquad (9)$$

The need for the flavins to mediate electron transfer from NADPH to heme may be severalfold. Certainly, one reason is that heme requires single electrons at different stages of NO biosynthesis and the flavins can serve as one-electron donors (Scheme 1), whereas NADPH, an obligate two electron donor, cannot. Furthermore, the oxidation of Arg to NO has been shown to require 1.5 NADPH (3 electrons) per NO synthesized, yet NADPH can only supply an even-numbered amount of electrons per catalytic cycle. Therefore, to accomplish the oxidation, NOS appears to be able to store an "odd" electron that is donated by NADPH between successive rounds of NO synthesis. Since both FMN and FAD can each accept and retain up to two electrons from NADPH, they can serve as the electron store between catalytic cycles. Therefore, in one cycle of NO synthesis, 2 NADPH would be used, with the fourth electron donated by NADPH being stored by the flavins for the next round of NO synthesis. In the second cycle the stored electron from the previous round of NO synthesis would be used along with two more electrons supplied by an additional NADPH. Therefore, each round of NO synthesis from Arg would use the experimentally determined 1.5 NADPH per Arg to NO cycle. Indeed it has been reported that the flavins of a rat liver microsomal cytochrome P_{450} reductase are capable of storing electrons between oxidation cycles.[58] A proposed scheme for NADPH and flavins usage in two successive rounds of NOS-mediated NO synthesis is depicted in Scheme 2. Although this scheme formally accounts for the number of electrons donated by NADPH and the pos-

Scheme 1.[a] Structures of the Oxidized, Radical, and Reduced Forms of Flavins



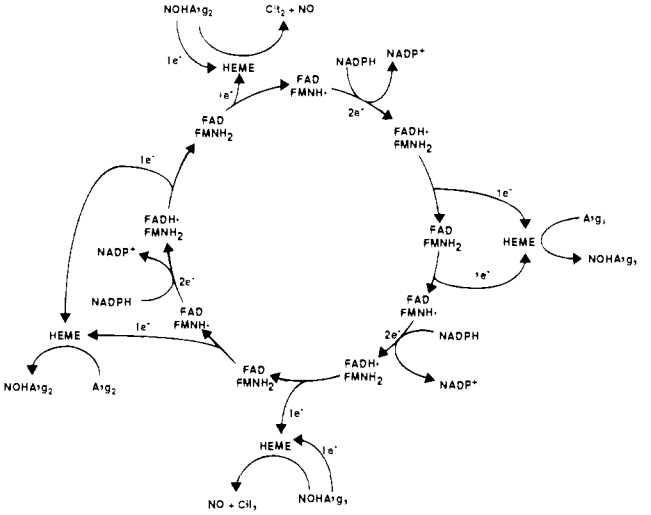
[a] Flavins can exist in one of three oxidation states. Upon one-electron reduction of the fully oxidized flavins (FMN, FAD), a semiquinone radical is obtained (FMNH•, FADH•), while a two-electron reduction generates the fully-reduced species ($FMNH_2$, $FADH_2$).

sible role the flavins have in storing electrons between successive cycles of NO synthesis, there is no proof that under physiological conditions the flavins cycle as shown. In an alternative mechanism the flavins may be maintained in one or more reduced states by a steady influx of electrons derived from NADPH. The subsequent transfer of electrons from the flavins to the heme would be coupled to Arg oxidation and thus account for the stoichiometry determined for NADPH oxidation. This latter mechanism for control of electron flow in NOS necessitates the substrate–heme complex regulate the stoichiometry of NADPH oxidation.

On the basis of the data accumulated on NOS and its similarity to a hybrid cytochrome P_{450} reductase–cytochrome P_{450} oxidase system, a two-step mechanism for the biosynthesis of NO from Arg has been proposed.[56,61] The oxidation of Arg to NOHArg at the heme site of NOS requires two electrons, both supplied by NADPH, to activate oxygen and then oxidize Arg to NOHArg (Scheme 3). The mechanism for this reaction is envisioned to be similar to that proposed for the oxidation of benzamidine to *N*-hydroxybenzamidine by cytochrome P_{450}.[62] The reaction likely proceeds by an initial perferryl heme, $[FeO]^{3+}$, mediated hydrogen atom abstraction of one of the equivalent Arg guanidino N-H's to form a guanidium radical cation. Recombination of the radicals, $[FeOH]^{2+}$ and guanidium radical cation, yields NOHArg and ferric heme.

The second step, conversion of NOHArg to NO and Cit, has no obvious precedent in other biological systems. An interesting aspect of the reaction is the necessity to invoke an electron transfer from an NOHArg-bound intermediate to the heme to allow for agreement with the observed overall NADPH stoichiometry as well as to provide the means to generate the odd-electron product, NO.[29] To account for the electron transfer, it has recently been suggested that a hydrogen atom transfer from NOHArg to the peroxyheme, $[FeOO]^{2+}$, is the most likely possibility based on redox potential measurements with NOHArg and thermodynamic considerations.[63] It is possible that the one-electron oxidation of the substrate occurs at some other point in the biosynthesis of NO; however, at this time it is not known exactly when NOHArg, or a subsequent intermediate, is oxidized. The mechanism depicted in Scheme 3, although very similar to mechanisms previously disclosed,[56,61] differs slightly in that it takes into account the possibility that NOHrg donates an electron to the enzyme via a hydrogen atom abstraction process.

Scheme 2.[a] Utilization of FAD, FMN, and NADPH in NOS-Mediated NO Synthesis[59]

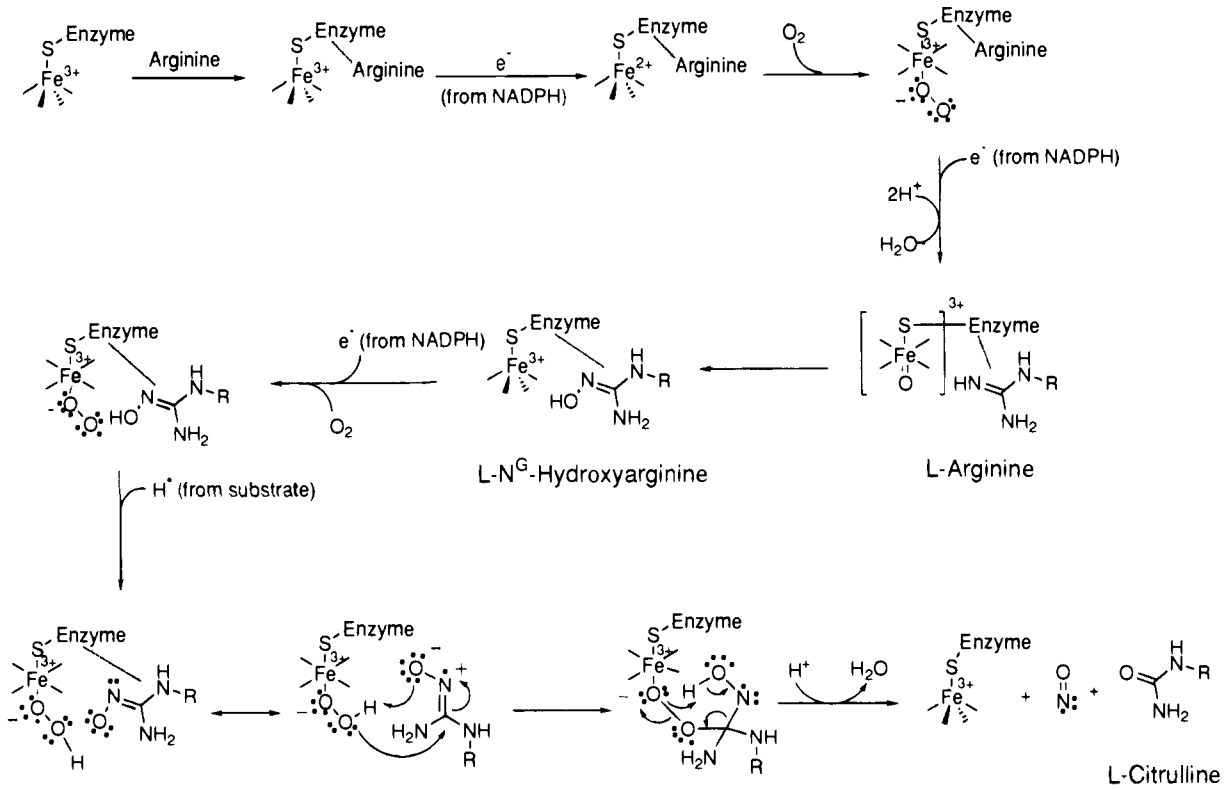


[a] In dual flavin enzyme systems there are five oxidation states possible for the flavins (0, −1, ..., −4). NOS-I and -II are isolated containing one electron in the flavin-containing domains.[60] In dual flavin enzymes similar to NOS, the reduction potentials of the flavins are controlled by the proteins such that the FMN potential is always held more positive than FAD. Thus, electrons will flow from FAD to FMN. In the catalytic cycle shown, the resting enzyme contains FMNH•/FAD. Throughout the cycle the flavins accept two electrons at a time from NADPH and deliver one electron at a time to the heme. Two electrons from NADPH are used to oxidize Arg to NOHArg in each cycle. The oxidation of each NOHArg to NO requires one electron from NADPH and one electron from NOHArg. After the first cycle is complete the flavins have an "odd" NADPH-derived electron stored ($FMNH_2$, FAD) which is used in the next cycle of NO synthesis. Overall, two NO molecules are synthesized from two Arg and three NADPH are oxidized.

Another interesting aspect of the hypothesized mechanism is that in the conversion of Arg to NOHArg the oxidizing species is the electrophilic $[FeO]^{3+}$, whereas in the transformation of NOHrg to NO and Cit a nucleophilic peroxoiron species, $[FeOOH]^{2-}$, is invoked. Although proposing two distinct oxidized heme intermediates to oxidize Arg and NOHArg at first seems unsettling, it has been suggested[56] that such a mechanism is reasonable based on a dual-oxidation mechanism which has been proposed for the P_{450} enzyme aromatase.

One of the reasons NOS may use different oxidizing agents for Arg and NOHArg is a result of the two substrates having remarkably different chemical reactivities. NOHArg can be readily oxidized via hydrogen atom abstraction to yield ONArg,[63] a species that will likely be extremely electrophilic. Indeed, NOHArg is very electrophilic and is known to be rapidly converted to Cit at 23 °C in **1** N NaOH,[53] whereas Arg is stable under these conditions. Furthermore, model studies with the hydroxyguanidine, *N*-(*N*-hydroxyamidino)piperidine (NHAP), have demonstrated that nucleophilic peracids can oxidize NHAP to the corresponding urea

Scheme 3.[a] Proposed Mechanism of Nitric Oxide Biosynthesis by Nitric Oxide Synthase



[a] The conversion of Arg to NOHArg likely proceeds via a typical mixed-function monoxygenase reaction. Arg binds near the ferric heme, and an electron from NADPH is transferred to heme via the flavins, enabling dioxygen to bind. Transfer of a second NADPH-derived electron to the heme leads to scission of the oxygen–oxygen bond, with one atom of oxygen being released as water and the other remaining bound to form an oxoiron oxidant. This electron-deficient oxygen is inserted into the terminal guanidino N–H of Arg, reforming ferric heme and generating NOHArg as an enzyme-bound intermediate. The second step of the reaction involves a three-electron oxidation of NOHArg. An NADPH-derived electron is again transferred to the ferric heme, and a second molecule of dioxygen binds. The ferric-oxy heme acts as an oxidant by removing the hydrogen atom from the NOH function of NOHArg. The peroxoiron species carries out a nucleophilic attack on the ONArg radical ultimately yielding NO, Cit, water, and ferric heme.

and nitrogen oxide products at ambient temperature.[64] Although these same experiments have not been done with the corresponding guanidine, it is likely that the guanidine is much less reactive toward peracids. On the basis of these observations it is conceivable that in the NOS active site, the nucleophilic $[FeOOH]^{2+}$ will rapidly react with ONArg before the peroxoiron species can react further to generate $[FeO]^{3+}$. Thus, the chemistry of the heme at the active site of NOS does not change throughout the course of NO biosynthesis. Rather $[FeOOH]^{2+}$, an intermediate on the path to $[FeO]^{3+}$, is intercepted in the second part of the oxidation process by the electrophilic ONArg. Although all of the biochemistry data collected thus far on NOS are consistent with Scheme 3, alternative mechanisms cannot be discounted.

By analogy with substrate-induced heme activation in cytochrome P_{450} reductase/cytochrome P_{450} enzyme systems, Arg-mediated transfer of electrons from NADPH to the flavins and ultimately to heme in NOS is a reasonable hypothesis. However, Ca^{2+}/calmodulin binding at the central portion of NOS-I serves as an allosteric mechanism that aligns the C-terminal reductase with the N-terminal oxidase domains and promotes electron transfer from flavins to the heme.[65] When the Ca^{2+}/calmodulin complex is unbound, the domains are not aligned and electron transfer cannot occur. This mechanism of regulation of electron transfer by Ca^{2+}/calmodulin elegantly illustrates how NOS-I and -III are regulated by intracellular Ca^{2+} fluxing. Increasing intracellular Ca^{2+} levels results in formation of the Ca^{2+}/calmodulin complex which binds to NOS-I or -III and initiates NO biosynthesis.

Both rat cerebellar NOS-I and murine macrophage NOS-II are homodimeric in their catalytically active forms. The monomers of murine NOS-II are able to bind FAD, FMN, and NADPH, yet they cannot bind BH_4, heme, or Arg. Upon co-incubation of purified NOS-II monomers with heme, BH_4, and Arg, the catalytically active dimer forms with heme and BH_4 binding during assembly.[66] Limited trypsin proteolysis of dimeric murine NOS-II generates two fragments of 56 and 74 kDa. The 56 kDa fragment is homodimeric (112 kDa) in its native form and contains heme and BH_4. The 74 kDa fragment is monomeric in its native state, contains FAD, FMN, and CaM, and can bind NADPH. That the oxygenase fragment is dimeric and the reductase piece monomeric in its native states suggests that the NOS monomers dimerize in a head-to-head alignment with the oxygenase domains forming the dimeric structure and the reductase domains existing as independent arms.[67] Limited tryptic digestion of rat NOS-I yielded primarily two fragments of 79 and 89 kDa. The 79 kDa fragment contains the reductase domain, and the 89 kDa portion contains the oxygenase domain.[68] Studies are ongoing to clone and express these NOS-I fragments which will be used to further characterize these domains. Furthermore, expression of these smaller fragments should make NOS more amenable to structural analysis.

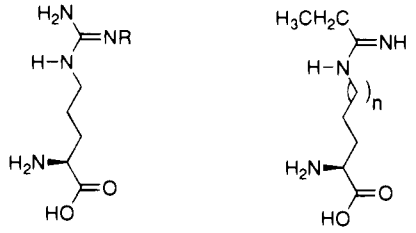
NOS Inhibition

The overproduction of NO in vivo is a potential pathophysiological mechanism in a variety of disease states. Accordingly, specific NOS inhibitors offer an opportunity for drug discovery. NMA (1) was the first NOS inhibitor shown to prevent the Arg-dependent macrophage-mediated synthesis of nitrite and cell killing of tumor cells.[33] Considerable effort has been expended to understand how NMA and other Arg analogs inhibit NO synthesis and to discover novel potent selective NOS inhibitors. The need for selective, bioavailable, specific NOS inhibitors cannot be understated. Given that NO is difficult to detect in biological systems, NOS inhibitors are extremely valuable in helping determine the presence of NO in a cell or tissue. Furthermore, NOS inhibitors are invaluable in delineating the role NO may have in the physiology or pathophysiology of a biological system.

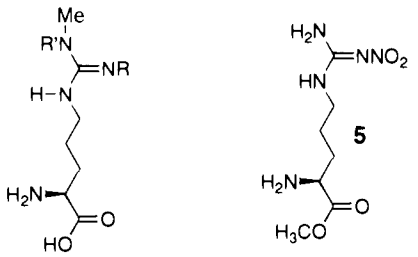
There are several approaches which have been explored to block NOS-mediated NO biosynthesis. Nonspecific calmodulin antagonists have been reported that inhibit electron transfer within NOS and, thus, prevent NO synthesis.[29] Indeed, given the tight, specific interaction of the Ca^{2-}/calmodulin/NOS-II complex, a selective inhibitor of this binding may offer a unique mechanism to regulate the NOS-II isozyme. Although the specific role BH_4, a tightly bound prosthetic group of all NOS isozymes, has in supporting NOS-mediated NO biosynthesis remains to be clearly elucidated, BH_4 analogs can compete for BH_4 and inhibit NO synthesis.[69] Given that tight BH_4 binding to NOS seems to be unique versus the role that BH_4 typically has as a recyclable prosthetic group in other enzymes, selective inhibition of NOS–BH_4 interactions may be feasible. Inhibition of NOS-II transcription may also offer an avenue to selectively control NO synthesis, but a greater understanding of the NOS-II promoter genomic sequence and transcription factors needed for NOS-II transcription is required before the rational inhibition of NOS-II via this mechanism is realistic. Several of these approaches, as well as others, for inhibiting NOS have recently been reviewed.[36,70]

The most promising approach to selectively inhibiting NOS isozymes to date has been with Arg-based or active site inhibitors. The prototype Arg analogs that have seen extensive use as inhibitors of NOS (see Figure 3) are NMA (**1**), L-N^G-nitroarginine (NNA, **2**), L-N^G-aminoarginine (NAA, **3**),[71] L-(iminoethyl)ornithine (NIO, **4**), and L-N^G-nitroarginine methyl ester (NAME, **5**).[70] NAME has been used a great deal, especially in cell assays, because it is more lipophilic than the other Arg analogs and can more readily penetrate the cell membrane. NAME is subsequently hydrolyzed by intracellular esterases to NNA which is the active metabolite.[72] NNA is unique among the prototype Arg analogs as it is 300-fold selective at inhibiting bovine NOS-I versus murine NOS-II.[73]

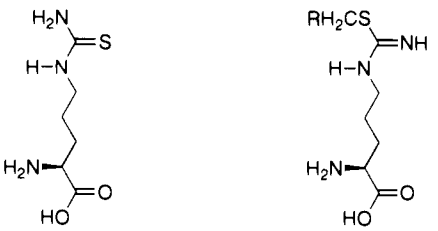
NMA, a non-selective NOS inhibitor, is a mechanism-based irreversible inhibitor of rat NOS-I and murine NOS-II isozymes.[61,74] The irreversible inactivation of both NOS isozymes requires, in part, an NADPH-dependent hydroxylation of NMA to L-N^G-hydroxy-N^G-methylarginine (NOHNMA, **6**).[61,74] A few mechanisms and metabolites for the NMA-mediated NOS inactivation have been proposed.[61,74] Recent data using two differentially radiolabeled NMAs suggest that the inactivation of NOS involves two or more NMA/NOHNMA metabolites.[75] NIO[76] has also been reported to be an irreversible inhibitor of NOS.[70]



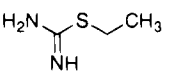
1 R=Me, L-N^G-methylarginine **4** n=1, L-iminoethylornithine
2 R=NO_2, L-N^G-nitroarginine **9** n=2, L-iminoethyllysine
3 R=NH_2, L-N^G-aminoarginine



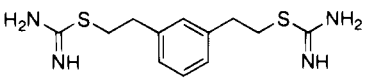
5 L-N^G-nitroarginine methyl ester
6 L-N^G-hydroxy-N^G-methylarginine R = H; R' = OH
10 L-N^G-methyl-N^G-methylarginine R = H; R' = Me
11 L-N^G-methyl-$N^{G'}$-methylarginine R = Me; R' = H



7 L-thiocitrulline **8a** L-S-methylisothiocitrulline R=H
8b L-S-ethylisothiocitrulline R=Me



12 S-ethylisothiourea



13 S,S'-(1,3-phenylenebis(1,2-ethanediyl))bis isothiourea

Figure 3. Structure of NOS inhibitors.

Newer classes of NOS inhibitors include thiocitrulline (**7**), an Arg competitive inhibitor of both NOS-I and -II with K_i values in the low micromolar range that has been shown to be a potent pressor agent in normal and endotoxemic rats.[77–79] In addition to competing for the Arg binding site, thiocitrulline (**7**) and NNA (**2**) also specifically inhibit electron transfer from the flavins to the heme.[78,79] For thiocitrulline (**7**) it has been hypothesized that the sulfur atom of the thiourea group interacts with the heme iron of NOS and reduces its reduction potential, thus making electron transfer from the flavins to heme less favorable. The oxygen of the nitro group of NNA (**2**) is likely functioning in a similar manner as the sulfur atom of the thiourea group of thiocitrulline. These inhibitors offer a novel mechanism

of inhibition of NOS activity which may be extended by using other heme iron ligands on Arg templates.

S-Methyl- and *S*-ethyl-L-isothiocitrulline (**8a**, Me-TC; **8b**, Et-TC) are potent, reversible, slow-binding inhibitors of all NOS isozymes.[80] These compounds were more potent at inhibiting human NOS-I versus NOS-II and -III. Et-TC inhibits NOS-I with a K_i value of 0.5 nM compared to 20 and 24 nM versus NOS-II and -III, respectively. Although Me-TC and Et-TC have excellent potency in the purified enzyme assays, the compounds were much less potent when tested in a cell-based enzyme assay. Et-TC is 15-fold less potent at inhibiting Cit formation in rat brain slices than rat brain cytosol, presumably due to poor cell penetration.

L-N^G-(1-Iminoethyl)lysine (NIL, **9**) is a selective inhibitor of murine macrophage NOS-II (IC_{50} value = 3.3 μM) versus rat brain NOS-I (IC_{50} value = 92 μM).[81] Interestingly, an analog of NIL which is one methylene group shorter, NIO (4), also inhibits NOS with equivalent potency but no isozyme selectivity. The subtle change in structure between NIL and NIO thus has a remarkable effect on isozyme selectivity.

NMA, L-N^G,N^G-dimethylarginine (ADMA, **10**), and L-N^G,$N^{G'}$-dimethylarginine (SDMA, **11**) have been isolated from mammalian sources,[82] leading to the speculation that these methylated Args may represent endogenous inhibitors of NOS activity. Levels of endogenous NOS inhibitors have been linked to chronic renal failure.[83] Methylated Arg also appears in proteins and is formed by postranslational methylation of Arg in peptides.[84] The interplay of Arg metabolism may be complex since inhibition of enzymes which would metabolize ADMA or SDMA could provide localized concentrations high enough to potently inhibit NOS.[85]

Non-amino acids, such as aminoguanidine (IC_{50} = 5–30 μM) and methylguanidine (IC_{50} = 1000 nM), inhibit the NOS-II isozyme selectively in cells, tissues, and whole animals.[86,87] Unlike the Arg-based analogs little is known about the mechanism of aminoguanidine-mediated NOS inhibition nor the basis for its NOS-II isozyme selectivity.

Recently, potent and selective inhibition of NOS isozymes has been achieved with simple nonamino acid isothioureas and bisisothioureas.[88] *S*-Ethylisothiourea (Et-ITU, **12**) inhibits human NOS-I, -II, and -III isozymes with K_i values of 29, 19, and 39 nM, respectively.

The ITUs are competitive with Arg. The optical difference spectrum of murine NOS-II with Et-ITU was similar to the NOS-II/Arg difference spectrum. Both Arg and Et-ITU complexed with NOS-II show a peak in the absorbance spectrum at approximately 385 nm, a trough at 420 nM, and an isobestic point at 410 nM (type I spectrum). The type I spectrum obtained with NOS-II and either Arg or Et-ITU is similar to the spectra obtained with cytochrome P_{450} enzymes with substrates bound and is suggestive of Arg and Et-ITU perturbing the heme environment of NOS. It has also been suggested that Arg binding to NOS results in a low-spin to high-spin heme iron transition in a subpopulation of the enzyme that was not initially high spin.[89] On the basis of these experiments and the structural similarity with the guanidine group of Arg, it was hypothesized that the ITUs are binding at the guanidine portion of the substrate site.[88]

Although the simple ITUs did not show any isozyme selectivity, the bis-ITU *S*,*S*′-(1,3-phenylenebis(1,2-ethanediyl))bisisothiourea (**13**) had K_i values for human NOS-I, -II, and -III of 250, 47, and 9000 nM, respectively. The selectivity for NOS-II versus -III inhibition is thus nearly 200-fold. Also, both the ITUs and bis-ITUs showed 2–7-fold greater potency at inhibiting murine macrophage NOS-II versus human NOS-II isolated from human colorectal adenocarcinoma cells (DLD-1) cells. Interestingly, the opposite trend was observed with the Arg analogs NMA, NNA, and NIO. These amino acid inhibitors were 2–7-fold more potent against the human versus rodent NOS-II isozymes. These results are suggestive of potential species differences which may be found as NOS inhibitors become more structurally diverse.

Disappointingly, neither S-ITUs nor bis-ITUs were very potent at inhibiting NO production in human cells. The ratio of the potencies for inhibiting NOS-II in DLD-1 versus purified NOS-II was approximately 600 for S-ITU and 3000 for **13**. Although the limited cell penetration of the ITUs and bis-ITUs makes them poor candidates for cellular or animal studies, the demonstration that potent, selective NOS isozyme inhibition is feasible with non-amino acid compounds is extremely encouraging and should stimulate continued studies.

On the basis of the results obtained with NOS substrates and amino acid- and non-amino acid-derived inhibitors of NOS, a pictorial model of the NOS active site with these compounds bound has been proposed.[88] On the basis of the structural similarity of the inhibitors to Arg, or a portion of Arg, coupled with the spectroscopic data obtained with some of the inhibitors bound to NOS, it is likely that these inhibitors bind to the NOS active site. The proposed binding of the NOS substrates Arg, NOHArg, and NMA to the enzyme active site is depicted in Figure 4a. The proposed critical regions of the NOS active site are the amino acid, guanidinium, and heme regions. Figure 4b shows several different inhibitors bound to NOS. Interestingly, the isothioureas are potent inhibitors of NOS, yet they lack the amino acid functionalities found in all Arg-based inhibitors. Recently, the natural product amino acid, L-indospicine (**14**),[90] and amino acid **15**[80,90] were synthesized and tested as inhibitors or substrates of NOS. Both compounds bind very poorly to NOS, suggesting that the guanidino nitrogen linked to the amino acid chain may be involved in a critical hydrogen bond to the backbone of NOS. Therefore, the original model for substrates and inhibitors bound to NOS has been extended in Figure 4a–c to include a hydrogen bond accepting group as part of the guanidinium binding site.[90] On the basis of these data, a compound that can bind both the heme moiety and guanidinium binding site seems to be sufficient to potently bind to NOS.

There exists an indirect regulatory mechanism that often is overlooked in the discussion of NOS inhibitors and Arg analogs—Arg transporters. Arg is obligatory for NOS activity, and one means of regulating Arg intracellular levels is via active transport of Arg. The predominant transporter for Arg cellular uptake is the y^+ transporter, which transports cationic amino acids. In J774 murine macrophages the y^+ transport system mediating Arg and NMA uptake is upregulated by cytokine treatment, while a neutral transporter mediating uptake of Cit, NNA, and NAME is insensitive to LPS induction.[91] Thus blockade of Arg transport systems would indirectly limit NO production in cells that

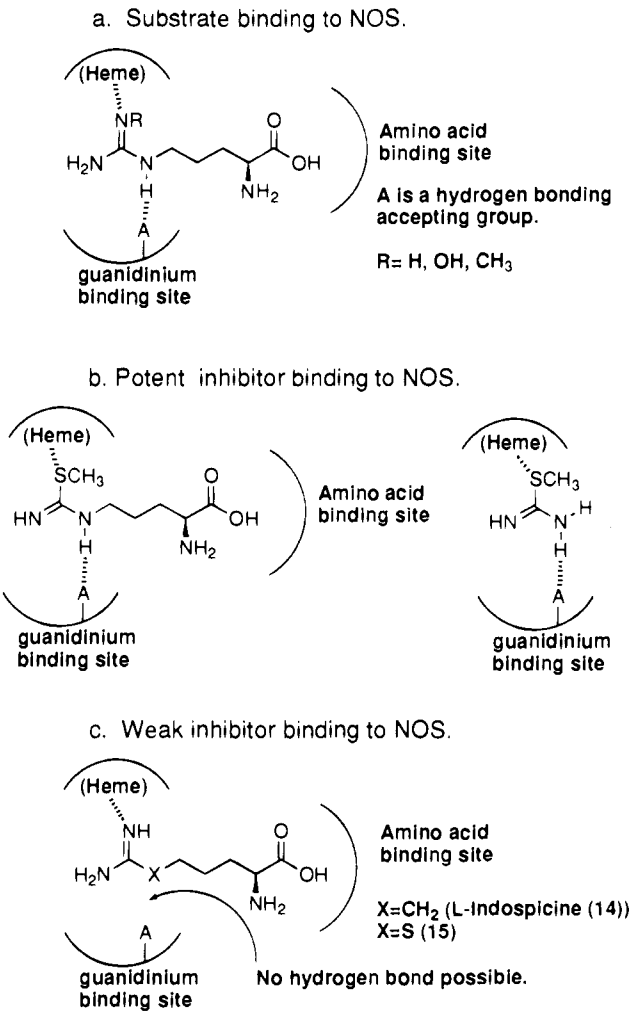
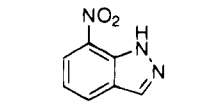


Figure 4. Proposed model of binding of NOS substrates and inhibitors to NOS.

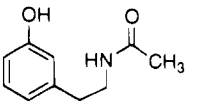
require import of extracellular Arg for substrate. To date several NOS inhibitors (NMA, NIO) have been shown to compete with Arg for the y^+ transporter, and this competition may in fact appear to potentiate the potency of these inhibitors by limiting pools of Arg available as substrate. However, no agent to date has been demonstrated to inhibit NOS activity via this mechanism.

Another endogenous regulator mechanism is the feedback inhibition of NOS by NO itself.[23] NOS inhibition by NO and other heme ligands is consistent with the evidence that heme is involved with the oxidation of Arg. The potential importance of this effect has been documented with clinical use of NO inhalation in pulmonary hypertension.[92] In vitro NO inhibition of NOS is attenuated by BH_4, suggesting a putative role for BH_4. This effect may have relevance since increased activity of NOS-III has been demonstrated by cytokine treatment which increase BH_4 levels, even in the presence of falling levels of functional protein.[93]

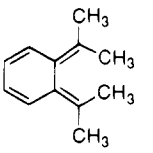
7-Nitroindazole (7-NI, **16**; Figure 5) inhibits NOS-I ($K_i = 0.16\ \mu M$), and the inhibition of Cit formation was found to be competitive with BH_4 and with Arg. However, the inhibition of NOS-II ($K_i = 1.6\ \mu M$) was found to be noncompetitive with substrate.[94] Since 7-NI competes for both Arg and BH_4 sites, it has been suggested that the BH_4 binding site, which allosterically interacts with the substrate–heme locus, may be proximal to the bound heme.[95] This suggests the possibility of NOS isoform selectivity based on interaction of the



16 7-Nitroindazole



17



18 7,7,8,8,-tetramethyl-o-quinodimethane

Figure 5. Structure of NOS inhibitors acting at different sites than L-arginine inhibitors.

inhibitor with multiple domains. In vivo, 7-NI inhibits NOS-I activity and possesses antinociceptive properties in the mouse.[96] However, more recently 7-NI was found to relax smooth muscle in vitro via non-NOS-related pharmacology, suggesting more cautious interpretation of in vivo results.[97]

One approach to NOS-II inhibition has been the control of obligate increases in BH_4 by inhibiting BH_4 biosynthetic pathways and in particular GTP cyclohydrolase and sepiatrin reductase.[98,99] This approach has some merit for acute treatments since it may not directly interfere with normal BH_4 levels. Some interesting inhibitors of sepiatrin reductase such as **17** have been identified which appear superior to GTP cyclohydrolase inhibitors.[100]

Phosphorylation of NOS-III appears to control its cellular translocation and hence its reactivity to shear stress and hormonal triggering. There are several consensus sites for phosphorylation by PKA (NOS-I and -III), PKC, cGMP dependent kinase (NOS-I), and calmodulin kinase II in the cloned NOS. Experiments with calmodulin antagonists, calmodulin kinase II, PKA, and PKC inhibitors suggest that a calmodulin-dependent kinase other than calmodulin kinase II is responsible for the translocation of NOS-III in bovine aortic endothelial cells (BAECs).[101] In general, nonselective kinase inhibitors like staurosporin decrease activity of NOS-I while there does not appear to be any phosphorylative regulation of NOS-II. NOS is also a substrate for calcineurin, which increases NOS activity upon dephosphorylation.[102]

The direct trapping of product NO has been demonstrated particularly with 7,7,8,8-tetramethyl-o-quinodimethane (NOCT-**1**, **18**), a reagent designed for detecting NO.[103] A less exploited means of regulating NOS is the trapping of NO or NO-derived products such as peroxynitrite. Hemoglobin is a highly effective NO trap and cell free hemoglobin appears to scavenge NO perhaps via redox reaction from metHb back to oxyHb.[104] The therapeutic use of radical scavengers and/or reductive agents is not novel, and an application of this approach to peroxynitrite has been reported with cysteine and penicillamine derivatives.[105]

NO Signal Transduction Mechanisms

The most obvious effector pathway for NO is activation of sGC. sGC is a heterodimer containing a heme

unit as well as a copper ion, and the activation of sGC by NO is presumed to occur by displacement of the heme iron from the plane of the porphyrin ring as in protoporphyrin IX binding of NO.[106,107] Increased cGMP levels due to activation of sGC by NO have been demonstrated in several cell types such as smooth muscle cells, platelets, neurons, and astrocytes.

NO has been reported to enhance ADP ribosylation of proteins and in particular the ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This reaction is now thought to involve the intermediacy of a nitrosothiol formed at the active site cysteines and concommitant formation of an GAPDH–NO–NAD complex when β-nicotinamide adenine dinucleotide (NAD) is present. Since this effect has been observed in vitro and the GADPH–NO–NAD adduct has yet to be fully characterized, the physiological relevance of this NO–NAD modification remains to be addressed.[108] Poly(adenosine 5′-diphosphoribose) synthetase (PARS) has also been reported to be another target for NO-mediated ADP-ribosylation and NO-mediated neurotoxicity has been suggested to result from ADP ribosylation of PARS.[109]

Nitrosation and nitration of proteins may be an important but less well characterized signalling pathway of NO. NO regulates NOS via feedback inhibition, and the Ca^{2+} flux through the NMDA ion channel is also inhibited by NO in a feedback loop. S-Nitrosation of glutathione depletes intracellular glutathione and activates the hexose-monophosphate shunt pathway. NO inhibits thioester-linked long-chain fatty acid acylation presumably via nitrosation of cysteine thiol substrates.[110] Nitration of tyrosines via the intermediacy of peroxynitrite has been hypothesized to alter the metabolic fates of the nitrated proteins relative to their parent proteins,[15] and nitrated proteins may indeed be indicative of chronic inflammatory disorders.[111]

NO and nitroso-containing compounds have been shown to chemically modify DNA both in vitro and in vivo. The effect of NO on DNA may be mediated by inhibition of ribonucleotide reductase which provides deoxyribonucleotides for synthesis of DNA.[112] Ribonucleotide reductase contains a tyrosyl free radical in the β_2 subunit which is effectively scavenged by NO under aerobic conditions. NO is also a potent activator of poly(ADP-ribose) synthetase, which is only activated by damaged DNA.[109]

Cytotoxic Role of NO

NO production in response to immune activation or inflammatory reaction is a seminal part in the history of the development of the concept that mammalian cells endogenously synthesize nitrogen oxides.[113] Nitrite and nitrate were produced in murine macrophages in vitro in response to LPS.[32] Macrophage stimulation resulted in the formation of nitrosonium ion [NO^+] equivalents, as detected by N-nitrosation of morpholine,[9] and this indicated the formation of a reactive species which subsequently produced nitrite and nitrate.

In vitro, macrophages can be activated by treatment with LPS and interferon-γ,[113] and activated macrophages (AM) inhibit DNA synthesis in tumor cells within 2 h.[114] Tumor cells injured by AM are able to survive (although unable to proliferate) if glucose is present in the medium. Importantly, tumor cells not treated with AM survive and even proliferate in the absence of added glucose. Injury induced by AM is Arg dependent and characterized by inhibition of mitochondrial respiration, with consequent depletion of ATP. There is a dramatic loss of electron transfer through the mitochondrial electron transfer chain in AM-injured tumor cells that is confined to the initial segments of the chain (complexes **I** and **II**), which contain almost all of the nonheme iron–sulfur clusters.[115] AM-injured cells also lose a substantial amount of total intracellular iron (64%) that occurs at the same time as the mitochondrial inhibition.[116] Inhibition of DNA synthesis was suggested to result from loss of activity of the nonheme iron-containing enzyme ribonucleotide reductase, which catalyzes the rate-limiting step in DNA synthesis.[114]

Examination of AM-induced injury by electron paramagnetic resonance (EPR) spectroscopy reveals the formation of complexes of the general formula $(RS)_2Fe(NO)_2$,[117,118] which are also observed when nonheme iron–sulfur-containing enzymes are treated with NO.[22] By using isotopically substituted Arg it was shown that the nitroso groups in these complexes derived from the terminal nitrogen in the guanidinium group of Arg.[118] A significant portion of these complexes localize to the mitochondrial fraction of AM-treated tumor cells,[117,118] suggesting that NO can attack and destroy nonheme iron–sulfur centers of essential enzymes, which results in cellular toxicity. NO synthesis by AM results in nitrogen oxide species which are more reactive than NO. In vitro very reactive species are indeed formed from the reaction of NO with O_2 or $O_2^{\bullet-}$ and species, such as peroxynitrite, also play a role in immune system-induced cell and tissue damage.[119]

NO in Vascular Regulation and Homeostasis

The vascular endothelium is now regarded as an endocrine gland and not simply as an inert vessel. One of the most potent substances released by the vascular endothelium is NO. The second messenger is released by the action of several endothelium-dependent vasodilators which cause a rise in intracellular calcium leading to the activation of NOS-III. NO diffuses from the endothelial cell to the adjacent vascular smooth muscle cells activating sGC, producing cGMP, that then mediates further signal transduction and ultimately leads to vasorelaxation. NO dependent relaxation has been demonstrated in isolated arteries, veins, and microvasculature. Local infusion of NOS inhibitors into the brachial artery of humans has reduced blood flow by as much as 40%. Thus, resistance vessels are continually modulated by NO and NOS activity is responsible for the maintenance of a basal dilatory vascular tone that vasoconstrictors then modulate. Mechanical stimuli such as shear stress are also sufficient to evoke changes in intracellular calcium and NO synthesis which may act to minimize cardiac load by optimally dilating local systemic vasculature.[120]

In addition to its effects on smooth muscle within the blood vessel wall, NO also inhibits platelet aggregation via the sGC-cGMP dependent pathway. Together with prostacyclin, NO provides a defense against platelet aggregation and adhesion to the endothelium. The effect of NO on platelet aggregation has been observed in vivo using cyclic flow reductions (CFR) at the site of stenosis.[121] NO dramatically reduced CFR frequency and this effect was blocked by NMA. In vitro NO inhibits platelet aggregation induced by various stimuli

as well as induces disaggregation of aggregating platelets.[122] This effect extends to monocytes and neutrophils, although platelets appear more sensitive to the antiaggregation effects of NO. In vitro these effects are well characterized. In vivo the fate on NO is less understood. In whole blood nitrate, methemoglobin and nitrosohemeglobin are formed in varying ratios depending upon conditions. In addition nitrosated proteins are also formed and can act as carriers of NO that in effect prolong its normal half life.[122]

NO can modulate platelet aggregation and vasodilation and regulate cardiac load at a local level, thereby acting as an important autocrine homeostatic modulator for the vascular system. A natural consequence of impairment of the endothelium and the vascular NO system is disease or predisposition to disease. Increased LDL in hypercholesterolaemia initiates macrophage-mediated oxidative injury of the endothelium and impairment of the NO transduction system. Studies of acetylcholine infusions in the forearm of diabetic patients indicated a loss of endothelium dependent vasodilation response presumably due to increased superoxide anion which effectively scavenges NO.[123] Thus, perturbations of the endothelial cell either in structure or in chemical environment can impact the function of NO in vascular homeostasis.

NO in Synaptic Plasticity

NO does not fit the concept of a classical neurotransmitter or neuromodulator. It is synthesized in the nerves and is released upon neuronal activation, but there are no storage or formal uptake pathways, although NO is rapidly inactivated in situ. Under normal physiological conditions NO has been reported to mediate and/or maintain synaptic plasticity, both long-term potentiation (LTP) and long-term depression (LTD),[124] activate sGC, couples neuronal activity to cerebral blood flow, and facilitates the release of various neurotransmitters and hormones.[125] A target of NO in presynaptic neurons is ADP ribosyl transferase which can be activated by NO and requires the small GTP-binding protein, ADP-ribosylation factor.[126] A mechanism by which NO potentiates transmitter release may be via direct stimulation of synaptic vesicle release which is independent of Ca^{2+} flux and activation of sGC.[127] This enhancement of neurotransmitter release by NO may explain its role in LTP. While the electrophysiological evidence for the role of NO in LTP in the hippocampus is documented,[128] NOS-I staining in the hippocampal CA1 pyrimidal neurons was weak or absent. This paradox has apparently been resolved by a transgenic mouse deficient in NOS-I which was found to stain for NOS-III in CA1 hippocampal neurons.[129] Thus, NOS-III and not NOS-I appears to subserve the critical role in modulating LTP in the hippocampus. In the hippocampus, NO may also act in concert with other retrograde messengers such as arachidonic acid, platelet activating factor (PAF), or carbon monoxide (CO) to regulate synaptic plasticity.[130,131]

NO as a NANC Neurotransmitter

Gastrointestinal (GI) smooth muscle function is regulated by nonadrenergic noncholinergic (NANC) nerves which, when stimulated, elicit hyperpolarization of postjunctional smooth muscle membranes, the inhibitory junction potential (IJP) and thereby produce relaxation. Several studies[132] support NO as one mediator of NANC neurotransmission. In vitro studies with NOS inhibitors have reduced inhibitory effects of NANC neural transmission in human cerebral artery strips, and this effect was reversed by Arg addition, providing support for NO-mediated control of cerebrovasculature via a NANC mechanism.[133]

NO protects the mucosal lining of the gastrointestinal system.[134] NANC-derived NO evokes smooth muscle relaxation, supporting the physiological role of NO in the stomach and the gastrointestinal system. At the same time, gastric mucosal blood flow is increased by neural regulation to allow for adequate perfusion to protect the mucosa from gastric contents.[135] Thus NO is involved with the muscle relaxation, the hemodynamic control, and the cytoprotection of the gastric lining. When NO synthesis is impaired, the gastric mucosa become more susceptible to lesions and ulceration.[136] Mutant mice lacking NOS-I are viable and fertile but have a marked enlargement of the stomach and hypertrophy of the circular muscle layer.[137]

NO also mediates penile erection by relaxing smooth muscle in the corpus cavernosum.[138] In a primate model penile erection can be induced by intracavernosal injections of NO donors *S*-nitrosocysteine and SNP and acetylcholine which stimulates the formation of NO, resulting in dose-dependent increases in cavernosal pressure and penile length. The maximal cavernosal pressure attained was similar for all three agents, but the duration of action was significantly shorter with acetylcholine.[139] The increase in cavernosal pressure induced by acetylcholine was blocked by NOS inhibitors. Likewise penile erections in rat evoked by apomorphine or oxytocin were blocked by NOS inhibitors.[140] These findings provide support for the hypothesis that NO is a prime mediator of penile erection and that NO donors may be useful in the treatment of erectile dysfunction.[141]

NOS-I has been found in skeletal muscle. NO promotes skeletal muscle relaxation through the cGMP pathway, and it also modulates increases in contraction that depend on reactive oxygen intermediates.[142] In actively contracting muscle, increased production of reactive oxygen intermediates diverts NO away from sGC toward sulfhydryl targets such as those on the sarcoplasmic reticulum. S-Nitrosation and resultant thiol oxidation reactions would promote Ca^{2+} release and potentiate muscle contraction.

Therapeutic Directions for NOS Inhibitors and NO

As discussed NO has an important role in many different and interrelated physiological processes in neurotransmission, vascular regulation, immunomodulation, and cytostasis. An understanding of the involvement of NO in pathophysiology is also evolving. In some cases a depletion of NO or an attenuation of its effector system could exist such as in hypertension, angina and impotence; in others an overproduction of NO may be inappropriate such as in circulatory shock, sepsis, stroke, and inflammatory responses. A crucial aspect of NO and NOS regulation will be selectivity, whether it be pharmacological, temporal, or regional, and bioavailability, since many of the present NOS inhibitors discussed show a marked loss of activity and selectivity in moving from the in vitro enzyme assay to cell-based assays.

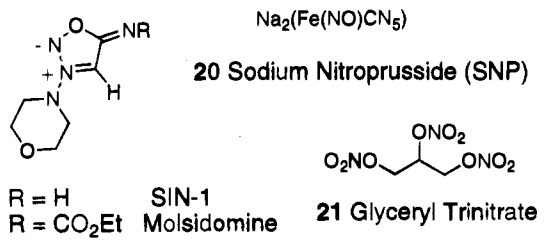


Figure 6. Structure of NO-donating agents.

Cerebrovascular Disorders: Cerebral Ischemia

Neurotoxicity in experimental models of cerebral ischemia/stroke has been shown to be attenuated by NOS inhibitors.[143] However, studies have been complicated because NOS inhibitors used also effect vascular NOS-III or regional cerebral blood flow. Therefore, conflicting results have been obtained. NAME was effective in reducing postischemic brain injury in the middle cerebral artery occlusion (MCAO) cat model when administered 45 min postinsult. Administration of an NO donor, SIN-1 (**19**) or sodium nitroprusside (**20**, SNP) (see Figure 6), in a focal ischemic model also resulted in attenuation of the infarct size and this effect was primarily attributed to an increase in cerebral blood flow.[144] Thus, NO has two divergent roles in focal cerebral ischemia: NO in the cerebrovasculature protects brain tissue via hemodynamic regulation whereas overproduction of NO is neurotoxic.[145] In a focal ischemia model in rat, NOS-I and NOS-I mRNA were upregulated after insult, suggesting that the increased NO arises from NOS-I.[146] Recent results in a transgenic mouse lacking NOS-I support the contention that NO derived from NOS-I is in part responsible for CNS tissue damage in cerebral ischemia.[147] Induction of NOS-II after cerebral ischemia has also been reported,[148] suggesting a possible role for NOS-II in post-trauma neurodegeneration. NOS-I is induced in spinal neurons following peripheral axotomy, which is suggestive of a greater role for NOS-I in peripheral nerve damage as well.[149] The role of NO in focal ischemia illustrates well the need for selectivity in the regulation of NOS. Hypothetically, one of the earliest responses to an ischemic insult is an increase in NO production presumably to augment cerebral blood flow. NO has also been proposed to down regulate NMDA receptor function by modifying the receptor protein—possibly through a disulfide–dithiol interchange sequence or by action at the redox modulatory site.[150] However, as NO levels build and cellular mechanisms for control of NOS are lost, NO plays a neurotoxic role which is further exacerbated as NOS-II is expressed.[125] During this phase NO may bind to ferritin, releasing iron that could contribute to lipid peroxidation.[151] NO also binds to iron responsive element binding proteins (IRE-BP), augmenting the activity of proteins under iron responsive element (IRE) control.[152] Therefore, strategies aimed at augmenting NO may benefit cerebral blood flow but also increase neuronal damage. Likewise, inhibition of NOS indiscriminantly in the evolving stroke could impair blood flow and also be detrimental.

The induction of NOS-II in astrocytes and microglia may be contributory to the CNS tissue damage that occurs in chronic neurodegeneration such as Alzheimer's disease[153] and multiple sclerosis since NOS-II has been identified in demyelinating regions in multiple sclerosis brains.[154]

NO and Migraine

NO has been suggested to be a major mediator of migraine and other vascular headaches.[155] Vascular headache produced by glyceryl trinitrate (**21**) has several chracteristics in common with migraine headache, including a pulsating and dose dependent headache. While migraine sufferers have been shown to be more sensitive to NO than control patients, it is not yet understood why migraine sufferers respond with more intense headaches. Since migraines are induced with both glyceryl trinitrate (**21**) and histamine administration, the difference is assumed to be downstream from NO production.[156]

NO in Other Vascular Disorders

In hypertension, endothelial dependent dilation is impaired even when blood pressure is controlled.[122] Endothelial dysfunction also impairs endogenous NO-mediated vasodilation in insulin dependent diabetes[122] and hypercholesterolemia.[157] Increased superoxide levels in both these conditions have been proposed to limit the amount of NO-mediated vascular response.[158]

Nitrovasodilators, including glyceryl trinitrate (**21**), SNP (**20**), and molsidomine (**22**), preferentially dilate veins because the sGC in venous smooth muscle is upregulated.[159] Similarly in vessels with damaged endothelium, sGC is upregulated and the vessel becomes more responsive to nitrovasodilation. This effect contributes to the antianginal profile of nitrovasodilators. Chronic inhibition of NO production has been shown to accelerate neointima formation in hypercholesterolemic rabbits,[160] suggesting that a role for NO in maintaining the patency of vascular systems. In arterial transplants (venous grafts) and angioplasty vascular endothelium may be damaged or produce insufficient NO and the lower amount of NO may not only contribute to the sensitivity of these vascular beds to nitrovasodilators but also contribute to vascular regrowth. NO possesses an antimitogenic effect on vascular growth and the absence of NOS in these regions may be a major factor in stenosis and restenosis.[161] GSNO has been successfully used to reduce angioplasty related platelet activation without altering blood pressure.[162]

NO and Atherosclerosis

Adhesion of macrophages to endothelium and subsequent formation of foam cells is an important event in atherogenesis.[163] Evaluation of the role of NO in macrophage adhesion and in atherosclerosis has indicated that while NO production is not decreased, activation of sGC is compromised. In patients with angina or coronary artery disease, vasodilatory responses to acetylcholine are impaired.[122] In the rabbit atherosclerotic aorta despite an impairment in endothelium dependent vasodilation, production of nitrogen oxides is enhanced rather than decreased and NO levels are increased in tissues from in vivo models of atherosclerosis.[163] The increased NO degradation products may contribute to cellular damage. NO may thus be cytoprotective because of its ability to scavenge superoxide anion,[164] and the decreased NO available may be insufficient to scavenge superoxide that is also present in atheromas. Increased superoxide levels have been suggested to account for diminished sGC responsiveness,[165] and these increased superoxide levels may

mediate the oxidation of low-density lipoprotein within atherogenic placques. Thus, a simple and perhaps slight imbalance in NO and superoxide may gradually contribute over time to atherogenesis. Loss of basal vasorelaxation on the basis of increased path length to sGC probably also contributes as the disease progresses.[2] In patients with hyperlipidemia, plasma Arg levels are 30% lower and this appears to become the rate-limiting aspect for NO synthesis. When Arg levels are increased, partial restoration of the acetylcholine dilator response results.[122]

Sepsis

Excessive production of NO can lead to vascular disease, and there is evidence that increased NO synthesis contributes to the vasodilation and hypotension observed in animal models of endotoxic shock or cytokine-induced shock. The increased NO results from induction of NOS-II and NMA has been shown to restore blood pressure and vascular responsiveness in animal models of endotoxic shock.[166] Clinical trials for endotoxic and sepsis have been conducted with the prototypical NOS inhibitor, NMA. Trials were based on the hypothesis that NOS-II, particularly in smooth muscle cells, is induced and leads to hypotension. Early clinical results suggested that low-dose NMA (0.3 and 1.0 mg/kg) did indeed reverse the hypotension; however, it also decreased cardiac output and heart rate which may have adversely effected peripheral tissue perfusion.[167] Further trials examining clinical outcomes of mortality and morbidity are required. Induction of NOS in myocytes and endocardium can also occur which may lead to depression of cardiac function; however, it remains to be proven whether this induction of NOS-II in the heart alters cardiac function in sepsis. Increased cytokine production is associated with liver failure and cirrhosis, and increased levels of NOS-II could also contribute to the lowered blood pressure and vasodilatation in these conditions.[122]

Besides an obvious drawback in the lack of NOS isoform selectivity with NMA, there are several other problems associated with clinical trials of NOS inhibitors in sepsis. Sepsis is a complex disorder with various stages of vascular response and organ failure, and consequently the regulation of NOS within the evolving septic episode needs to be considered.[162] Evidence suggests that early cytokine mediators actually down regulate NOS-III and impair vascular regulation quite early in sepsis.[168] At later times NOS-II may become more important, hence NO-based therapy may be appropriate early to maintain vascular perfusion and prevent leukocyte adhesion[169] while selective NOS-II inhibition may be desirable as sepsis progresses. Preliminary clinical results with low-dose NMA suggest that minimal inhibition of NOS may still lead to profound cardiovascular changes without a detrimental loss in physiological control of blood vessels. Furthermore the inhibition of NOS in sepsis will serve to protect vascular beds from NO-mediated damage.[122]

NO in Heart Failure

It is likely that NO is involved with intrinsic control of heart rate since NOS immunoreactive fibers and terminals are dense in the sinoatrial and atrioventricular nodes. Plasma nitrate levels are increased in patients with idiopathic dilated cardiomyopathy, and

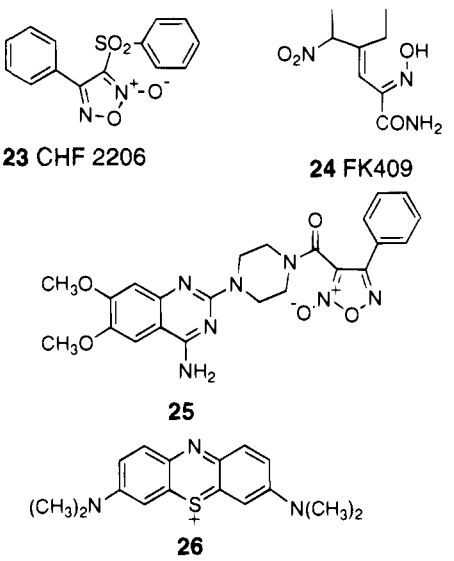


Figure 7. Structure of NO-donating agents and methylthionine (methylene blue).

NOS-II has been reported to be present in the myocardium of these patients. NO-mediated vasodilation may be a compensatory mechanism for a vasoconstrictor effect of neurohumoral adaptations to heart failure or excess NO may become detrimental by a direct negative inotropic effect.[170]

Transplantation

NO produced by NOS-II may play a significant role in the rejection of transplanted tissues.[171] However, NO produced during the reperfusion phase of transplantation may actual play a beneficial role. A study of small bowel transplantation in rats found a significant benefit to maintaining mucosal NO production within the graft during preservation of the tissue and particularly during reperfusion in the host.[172] When hosts were treated with NOS inhibitors (NAME and NMA), graft function and survival were impaired. When both donor and the grafted tissue were pretreated with NOS inhibitors, graft function was impaired but not survival.[172]

Rat mast cells produce NO in a constitutive manner and the concurrent elevation in cGMP levels appears linked to inhibition of histamine and superoxide release.[173] NO production in activated macrophages has been correlated with the inhibition of lymphocyte proliferation.[171] A similar effect was noted in allograft-infiltrating cells where proliferation was stunted by NO and could be enhanced by NOS inhibitors. Cyclosporin and FK-506 inhibit NO production in these mixed culture systems, allowing allospecific cytotoxic lymphocytes to develop and proliferate.

NO and NO Donors as Therapeutic Agents

As a result of the increased research in NO there has been a renewed effort to identify and therapeutically exploit new NO donors.[174] One example of this class are the NONOates which release NO in stoichiometric amounts under physiological conditions.[175] The half-lives of this class of NO donors is rather short-lived, but selective targeting of this class of agent has been demonstrated and further increases in residence times and selectivity are envisioned. CHF 2206 (**23**, Figure 7) is a furoxan with potent antiplatelet and vasodilating activity in vitro.[176] FK409 (**24**), a semisynthetic fermentation product, has also been characterized as a

potent NO donor with antiplatelet and vasodilatory properties. Furthermore, FK409 does not develop tolerance or cross tolerance with glyceryl trinitrate and does not require a sulfhydryl for NO release. FK409 appears to be well tolerated in humans after oral dosing and is currently being evaluated for the treatment of ischemic heart diseases.[177] While the significance of S-nitrosation in vivo is uncertain *S*-nitroso thiols possess the pharmacological and chemical properties of NO donors. Various *S*-nitroso thiol analogs of cysteine, penicillamine, and mercaptoethanol[178] have been prepared and evaluated for antiplatelet effects in vitro.[179] One of the more interesting exploitations of NO donors is the combination of an NO-donating moiety, a furoxan, with the α-1 blocking properties of the prazosin nucleus (**25**).[180]

NO in Pulmonary Hypertension

Perhaps the most surprising exploitation of the increased knowledge of NO and NOS is the use of NO inhalation therapy in pulmonary hypertension in neonates and adult respiratory distress syndrome (ARDS) patients.[181,182] While the direct application of a reactive species such as NO may initially appear foolhardy, it is the relative reactivity of NO which indeed makes this a very viable form of therapy. Currently low concentrations of NO (80 ppm and lower) are used, and the method has been found effective.[181] The use of NO gas mitigates any potential systemic vascular effects and limits vasodilation to those pulmonary arteries already receiving some degree of ventilation. Several issues have arisen in the practice of NO inhalation therapy. One problem that has apparently been solved is an accurate, fast, and safe way of monitoring the actual amounts of NO delivered to the patient,[182,183] reducing the likelihood that a patient may be receiving too much NO or NO_2. Other complicating factors are the initiation of NO inhalation therapy as a function of the phase of the disease process and the necessity of methylthionine (**26**) to treat methemoglobinemia resulting from therapy. Potential side effects related to increased bleeding times have been reported. Another more recently discovered problem is feedback inhibition of NOS by administered NO.[92] Nonetheless, NO inhalation is in a number of trials and appears to provide benefit for some patients.[182]

Penile Erectile Dysfunction

Muscles of the corpus cavernosum of the penis relax in response to stimulation of NANC nerves or NO-donating drugs to elicit erection. NOS is present in the pelvic nerve neurons ennervating the corpus cavernosum. NO has now been established as the physiological mediator of penile erection, and NOS inhibitors abolish evoked erections in laboratory animals and humans.[141] As a result there has been a renewed interest in exploiting NO-donor drugs for the relief of erectile dysfunction. Local injections or topical applications of nitrovasodilators has been effective in some cases. Current therapies inclusive of mechanical devices and locally injectable vasoactive agents are clearly not optimal, and the discovery of an orally active agent for penile erectile dysfunction would be a major breakthrough.[184]

Cytokine Regulation of NO in Disease States

Arg can evoke the release of insulin in the presence of glucose via a mechanism that can be blocked by NMA (**1**), suggesting that NO may be involved in insulin release under physiological conditions. Endothelium dependent vasodilation is also impaired in patients with type I diabetes and diabetic microangiopathy has also been suggested to be the result of defective endothelial NO production.[158] However, more recent results also suggest a role for NOS-II in the etiology of type I diabetes.[185] Pancreatic islets exposed to interleuken-1β (IL-1β) induce the synthesis of NOS-II and the resultant NO formation may play a role in the destruction of pancreatic β cells during the development of type I diabetes.[186] Islet cells were protected by NOS inhibitors. Cyclosporin A treatment, which can suppress the induction of NOS-II, also proved effective in preventing streptozocin-induced type I diabetes in the rat.[187]

NO in Inflammation

One of the prime functions of NO and its oxidation products during an inflammatory response is to promote microbial cytotoxicity. However, host tissues can also respond to NO, thereby altering physiological functions in the inflammed tissue. Systemic vasodilation as in sepsis can lead to severe hypotension. In skin and other tissues NO can cause local vasodilation and alter microvascular permeability leading to edema and erythema.[188] NO may be produced by different cell types and act in conjunction with other inflammatory mediators such as cytokines, arachidonic acid oxidative metabolites, histamine, and PAF. Tissue damage from NO may be indirect such as alterations in vascular perfusion rates and tissue edema or direct such as the reaction of NO with superoxide anion to produce peroxynitrite which subsequently promotes lipid and sulfhydryl oxidation.[188] Nitrogen oxides are commonly found in plasma and tissues samples derived from patients with chronic inflammatory conditions such as chronic colonic inflammation, ulcerative colitis, and arthritis.

A number of studies have shown attenuation of inflammatory responses with NOS inhibitors. Neurogenic airway edema in rats was prevented by NOS inhibitors.[189] Likewise in guinea pigs chronic ileitis induced by trinitrobenzenesulfonic acid was blocked by administration of NOS inhibitors.[190] Arthritis induced by intraperitoneal injection of streptococcal cell wall fragments in rats was reversed by NMA administration.[191] Adjuvant-induced arthritis in rats was reduced by NAME administration.[192] While it may appear that NOS inhibition may provide some relief from inflammatory disorders, a reduction in NO synthesis may also compromise a patient toward microbial infection. In addition, in the rat and the cat NOS inhibition promoted leukocyte adhesion in postcapillary venules and potentially this effect could oppose the antiinflammatory response of NOS inhibitors.

Chronic inflammation could lead to high localized concentrations of NO, NO_2, N_2O_3, and N_2O_4. Although normally at low concentrations, these nitrogen oxides may reach high enough localized concentrations in chronic inflammatory disorders to nitrosate amines and DNA, and this may be a plausible mechanism for the reported mutagenicity of NO.[188] This formation of nitrosamines in chronic inflammatory states could be involved with cancer etiology.

During prolonged inflammation or infection it is possible that Arg and/or BH_4 are depleted. Under these conditions NOS itself can become a source of reactive

oxygen free radicals as it reduces molecular oxygen to superoxide anion ($O_2^{\bullet -}$) and peroxide (H_2O_2).

Future Directions

NO research has increased dramatically since identification of EDRF as NO was first disclosed in 1986. NO may well be the first of a family of small molecules acting via changes in cellular metabolism and redox states rather than via traditional ligand–receptor interactions. Research on NO has already had a measurable impact in the practice of medicine with NO inhalation therapy and in the broadening of the understanding of disease mechanisms as in hypertension and septic shock. While the last half decade has seen an increase in NO research, there are still numerous unanswered questions and areas of less understanding such as the following: How does endogenously produced NO lead or lend itself to mutagenicity? What is the interrelationship between molecular oxygen, NO, and superoxide in normal homeostatic states? What role does NOS-II play in numerous disease pathologies? There is no doubt that continued research will shed more light on these and other issues. However, equally in need of breakthroughs is the area of selective NOS inhibition. NOS inhibitors known to date do not possess the desired pharmacological selectivity, pharmacokinetic and pharmacodynamic profiles to render them therapeutic agents. While the biochemistry and pharmacology of NO have advanced there has been less success in identifying highly selective inhibitors that can be used to probe the pharmacology of NOS. Clearly these selective agents will be needed not only as potential therapeutics but also as probes to allow new directions to emerge from the NO research field. The molecular biology of NOS has also advanced providing knock-out mice and numerous molecular probes. Continued research may well derive more NOS isoforms or a novel mode for regulation of NOS. Nitrovasodilators have been known for over a century, and with the reawakening derived from NO research the rational design of NO donors has taken on new meaning and vitality. Currently available nitrovasodilators may not be optimal for the various disease states with hypofunctioning NOS or perturbed NO signal transduction mechanisms. Novel NO-donating agents, in addition to being proprietary, could be designed with a specific therapeutic endpoint for optimal clinical benefit. There is much promise remaining in NO research and to date we have only glimpsed the tip of the iceberg.

Biographies

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