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Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization

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Abstract. Nitric oxide (NO) is a recently discovered mediator produced by mammalian cells. It plays a key role in neurotransmission, control of blood pressure, and cellular defense mechanisms. Nitric oxide synthases (NOSs) catalyze the oxidation of L-arginine to NO and L-citrulline. NOSs are unique enzymes in that they possess on the same polypeptidic chain a reductase domain and an oxygenase domain closely related to cytochrome P450s. NO and superoxide formation as well as NOS stability are finely regulated by Ca^{2+}/calmodulin interactions, by the cofactor tetrahydrobiopterin, and by substrate availability. Strong interactions between the L-arginine-metabolizing enzymes are clearly demonstrated by competition between NOSs and arginases for L-arginine utilization, and by potent inhibition of arginase activity by N^{ω}-hydroxy-L-arginine, an intermediate in the L-arginine to NO pathway.

Key words. L-Arginine; nitric oxide; nitric oxide synthase; arginase; urea cycle; N^{ω}-hydroxy-L-arginine; superoxide radical; peroxynitrite.

Introduction

In mammalian cells, the semi-essential amino acid L-arginine is involved in protein synthesis. It is also used as a substrate by enzymes like nitric oxide (NO) synthases (NOSs), arginases, arginine decarboxylase, or glycine transamidinase [1–3]. Among these pathways, the biosynthesis of NO and the urea cycle are presumed to play the most important roles in the metabolism of L-arginine (fig. 1). L-Arginine is metabolized by NOSs to form NO and L-citrulline, and by arginase to form urea and L-ornithine, a precursor of the polyamines spermine and spermidine used as growth factors. NO is an important mediator of many (patho)physiological events [3] whereas arginase is a key enzyme of the urea cycle, an essential metabolic pathway for the removal of highly toxic ammonium ions resulting from protein degradation [2, 4].

Many books and reviews summarize current knowledge concerning the roles of NO in (patho)physiological situations and its biosynthesis from L-arginine [5–8]. The present article will focus on some aspects of the biology and enzymology of NOSs and arginases. Readers interested in the transcriptional and translational regulation of these enzymes are directed to reviews that have appeared in the literature [8, 9]. Regulation of NO biosynthesis has been achieved using L-arginine analogs, able to compete for access to the active site. It now appears that regulation of NO formation can also be obtained by modulation of L-arginine availability, through the involvement of arginases. These results have led many research teams to investigate the cross-regulations between some of these L-arginine-metabolizing enzymes. There is currently a resurgence of interest in arginases in the light of their newly perceived roles in the regulation of NO synthesis.

Three isoforms of NOS

In mammalian cells, NOSs are the enzymes responsible for NO generation and they catalyze the oxidation of one N^{ω}-atom of the guanidino group of L-arginine to

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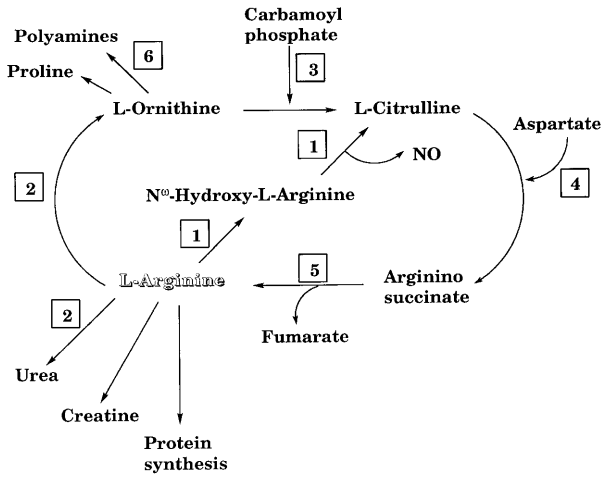

Figure 1. Metabolic pathways and enzymes related to L-arginine metabolism. Some of the enzymes involved are numbered as follows: 1 = nitric oxide synthase; 2 = arginase; 3 = ornithine carbamoyltransferase; 4 = argininosuccinate synthase; 5 = argininosuccinate lyase; 6 = ornithine decarboxylase.

NO and L-citrulline in two steps, with formation of the intermediate N$^{\omega}$-hydroxy-L-arginine (NOHA) [3, 5–7, 10] (eq. a, b, fig. 2). Three isoforms of NOS (NOS I, II, and III) are encoded by three different genes and can be classified into two families: constitutive NOS (NOS I and III) and inducible NOS (NOS II) [11–13] (table 1). The constitutive NOS I and NOS III were first characterized in neurons and in vascular endothelial cells, respectively, and they are finely regulated by the Ca^{2+}/calmodulin (CaM) complex. NOS I and III produce short-lasting (seconds to minutes) and small quantities of NO which have regulatory roles in neurotransmission and the cardiovascular system [3, 11–13]. NOS II was first isolated from murine macrophages stimulated with lipopolysaccharide (LPS) and interferon-γ. Due to its high affinity for CaM, NOS II is mainly Ca^{2+} independent and can produce NO over long periods (hours to days) after a delay required for protein synthesis. Indeed, it appears that CaM copurifies and forms a very tight interaction with inducible NOS. NO produced in this way is the main effector of the antiproliferative effect exerted by activated macrophages. Examples of the physiological settings in which these three isoforms operate are indicated in table 1. The distinction between the constitutive and inducible character of NOS is not clearcut, since induction of all three isoforms has been observed to some extent [14]. High sequence homologies are observed between similar isoforms from various species but lower homologies exist between each isoform within a species. Human and bovine NOS III display 93% homology and there is 80% homology between NOS II from murine macrophages and human hepatocytes, whereas human NOS I, NOS II and NOS III show only 50–60% homology [11–13]. However, the regions of higher homology are associated with common sites for the binding of cofactors and the coenzyme CaM.

NOS structure

Though the different forms of NOS vary slightly in their mode of expression and regulation, the overall reaction catalyzed is the same, a five-electron oxidation of L-arginine to form NO and L-citrulline (fig. 2). All the isoforms contain conserved amino acid sequences for the binding of NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in their C-terminal domain [11, 12]. This C-terminal domain shows a high degree of sequence homology to NADPH-cytochrome P450 reductase and is called the 'reductase domain' [15]. The N-terminal domain contains a heme-(iron protoporphyrin IX) binding site including a cys-

Table 1. Main properties of the three nitric oxide synthase (NOS) isoforms (in rat).

Isoform	NOS I (nNOS)	NOS II (iNOS)	NOS III (eNOS)
Type	constitutive	inducible	constitutive (but regulated)
Subcellular localization	cytosol	cytosol	membrane and cytosol
Cell, tissue	brain neuron lung kidney	liver hepatocyte kuppfer cell macrophage smooth muscle chondrocyte	endothelium epithelial cell
Regulation	calmodulin antagonists phosphorylation	cytokines (interleukin-1, interleukin-4) glucocorticoids	calmodulin antagonists palmitoylation myristoylation phosphorylation
Ca^{2+}/calmodulin dependence	++	no	++
K_m (L-Arginine) (μM)	2–5	2–15	2–30
Molecular mass of monomer (kDa)	160	130	135

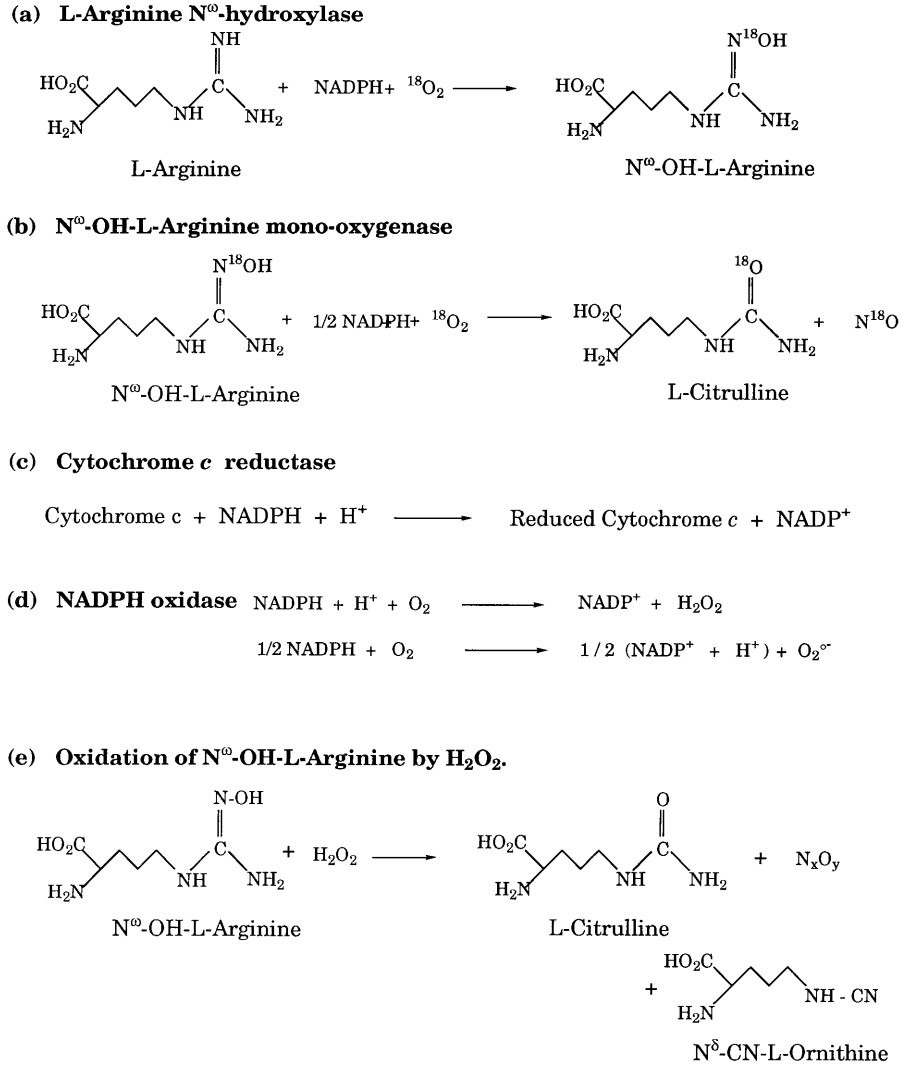

Figure 2. Reactions catalyzed by full-length nitric oxide synthases (NOSs).

tein residue as in cytochrome P450 [16–19]. Binding sites for the cofactor tetrahydrobiopterin (BH_4) and L-arginine are also present on the N-terminal domain, called the 'oxygenase domain'. This N-terminal part of the polypeptide chain contains serine and threonine phosphorylation sites, and in the case of NOS III, myristoylation and palmitoylation sites enabling interactions of this isoform with membranes [3, 5–7, 11–14]. The oxygenase and reductase domains of dimeric NOS I and NOS II have been cleaved by limited trypsinolysis and purified from one another [20, 21]. Recombinant reductase and oxygenase domains of the three isoforms as well as the corresponding 'full-length enzymes' can be expressed in yeast-, bacteria- or baculovirus-transfected cells [22–24].

NOSs are only active as homodimers of molecular weights between 250–300 kDa [11–14, 25] (table 1). CaM binding enhances the electron transfer in the reductase domain and allows heme reduction and NO synthase activity [26, 27]. Using the inducible mouse NOS isoform as an example, a general model for the stucture and domain composition of an active dimeric NOS has been proposed [28]. It shows two NOS II subunits aligned in a 'head-to-head' manner with the oxygenase domains of each subunit interacting together to form the dimer, and the reductase domains attached as independant extensions [28]. Recently, Siddhanta et al. [29] established that the reductase of one monomer transfers electrons from NADPH to the oxygenase-oxygenase interaction enables the enzyme to engage in a

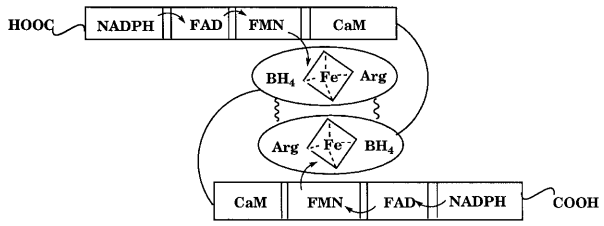

Figure 3. Proposed model for NOS II dimer organization with electron transfer pathway, oxygenase domain dimer formation and domain swapping between monomers [adapted from ref. 29].

type of 'domain swapping' which allows NADPH-derived electrons to transfer between reductase and oxygenase domains that are located on adjacent subunits (fig. 3) [29].

BH_4 plays a key role in NOS dimerization, L-arginine binding, and low- to high-spin transition of heme, allowing NOS activity [21, 30–32]. Purified NOS II monomers do not reassociate into a dimer when incubated together, but dimerization is promoted in the presence of L-arginine with BH_4 and a stoichiometric amount of heme [25]. However, significant dimerization of heme-free NOS I monomers is obtained upon addition of heme alone, suggesting that heme insertion is the main promoter of dimer assembly in this isoform and that differences could exist in isoform association [33–35].

Spectroscopic studies suggest strong analogies between the NOS oxygenase domain and P450-like heme-thiolate proteins. In the absence of both BH_4 and L-arginine, the ferric heme iron of NOS is predominantly six-coordinate low spin [20, 21, 31–35]. Binding of BH_4 and L-arginine occurs near the distal side of the heme and influences the binding of the other, but always favors the five-coordinate high-spin form [21, 31–35]. L-Arginine and BH_4 binding modify the binding of heme ligands such as CO, NO, O_2, nitrosoalkanes, and imidazoles to the ferric or ferrous heme of NOS [21, 36–41]. BH_4 shields some parts of the porphyrin macrocycle from alkylating agents generated within the active site [42]. In addition, BH_4 controls the redox properties of the heme [43]. BH_4 strongly modifies the midpoint potential value (E_0) of the inducible isoform but has few effects on the E_0 of the neuronal isoform, alone or in combination with L-arginine [43]. Recent crystallographic data on truncated NOS II oxygenase domains suggest that the guanidino group of L-arginine binds to the Glu371 carboxyl group which should position the guanidine directly above the heme. In addition, the same Glu371 could interact with the α-amino group of L-arginine (fig. 4) [44, 45]. These data suggest the binding of BH_4 almost perpendicular to the heme, in close interactions with a carboxyl group of the heme and blocking the access to the heme of even very small molecules like CO or NO [45].

The NOS enzymes thus behave as self-sufficient cytochrome P450s, with analogies to P450 BM_3 since they

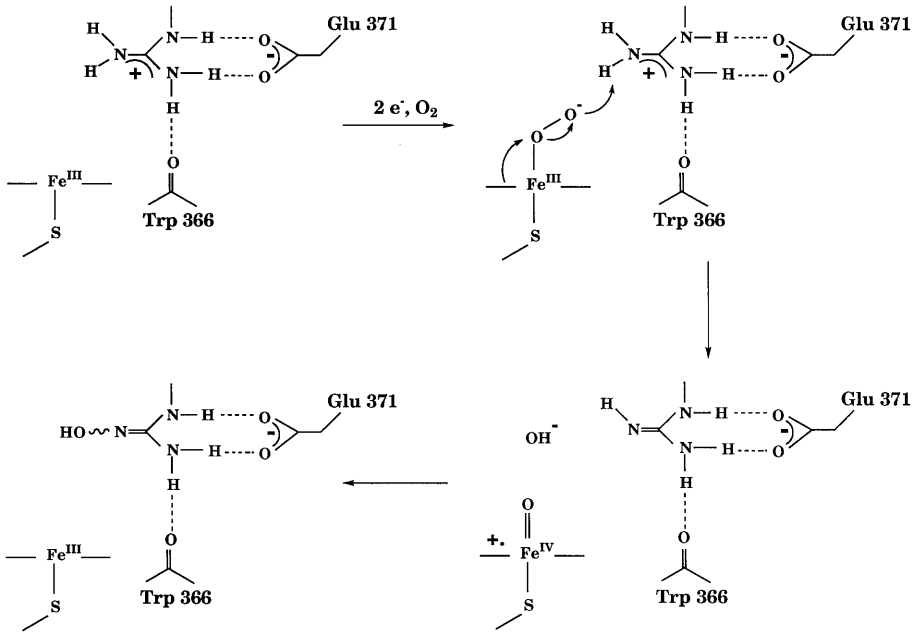

Figure 4. Proposed L-arginine assisted NOS oxygen activation [adapted from ref. 45].

bear their reductase and heme domains on the same polypeptide. However, they differ from 'classical' P450s by their requirement for the cofactor BH_4 for dimerization, heme transition, efficient electron transfer, and NO generation. Furthermore, the control of electron transfer from the reductase domain to the heme domain is finely regulated through binding of the coenzyme CaM between the two domains. Finally, they differ in the mechanism of the reaction leading to NO formation.

Mechanism of action of NOSs

Oxidation of L-arginine to L-citrulline and NO involves two steps with the formation of the intermediate NOHA [3, 5–7, 10, 21] (eq. a, b, fig. 2). Experiments using $^{18}O_2$ showed that the oxygen atom incorporated into NO and L-citrulline originates from molecular oxygen. The first step of the reaction (eq. a, fig. 2) leads to the formation of NOHA, an intermediate barely detectable in NOS-I-catalyzed reactions but which can accumulate in the reactional medium from several cell types possessing an active NOS II [46, 47]. In this first step, 1 mole of NADPH and 1 mole of O_2 are consumed, with concomitant formation of 1 mole H_2O. As in 'classical' P450-dependent N-hydroxylation of a guanidine, the high-valence iron-oxo species NOS-Fe^{v}=O is probably involved (reviewed in ref. [48]). However, based on the enhancement of the rate of catalysis by nonheme iron, Rusche et al. [49] recently connected NOSs to the known BH_4/nonheme-iron-dependent hydroxylases and suggested that a BH_4-O-OH active species could also perform this reaction.

The second step of NO synthesis (eq. b, fig. 2) involves the oxidative cleavage of the C=NOH bond of the intermediate NOHA and leads to NO and L-citrulline. It is less straightforward because it involves a three-electron oxidation of the NOHA hydroxylated nitrogen that is apparently coupled to the consumption of a second mole of dioxygen but requires only 0.5 moles of NADPH (eq. b, fig. 2) [3, 5–7, 10, 21, 50]. The mechanism is still controversial but probably involves the iron-peroxo intermediate (NOS-Fe^{II}-O_2↔NOS-Fe^{III}-O-$O^{\bullet}$) generated during the redox cycle as the key active species, with fragmentation of a tetrahedral intermediate to yield products in the last step [51, 52].

Based on crystallographic data, Crane et al. [45] recently proposed that formation of the high-valence species NOS-Fe^{v}=O could be favored by L-arginine itself (fig. 4). The active site of NOS would not possess an amino acid residue able to catalyze the cleavage of the Fe^{III}-O-O^{-} complex resulting in the NOS-Fe^{v}=O species, a reaction generally favored by a conserved threonine located near the active site of most 'classical' P450s [48]. At physiological pH, L-arginine is protonated and could assist the cleavage of the NOS-Fe^{III}-O-O^{-} complex to the NOS-Fe^{v}=O species, which is able to hydroxylate the N^{w}-atom of L-arginine (fig. 4). Differences in the pK values of NOHA compared to L-arginine (8.0 versus 13.0) could explain the involvement of the NOS-Fe^{III}-O-O^{-} complex in the second step of NOS rather than the NOS-Fe^{v}=O complex [45].

Monomeric or dimeric full-length NOSs as well as their isolated reductase domains catalyze electron transfer from NADPH to acceptors such as cytochrome *c* and ferricyanide at almost similar rates [21] (eq. c, fig. 2). The reductase domains are also able to transfer electrons to O_2 in both the CaM-free and CaM-bound states, then generating superoxide ions and H_2O_2 (eq. d, fig. 2), but with lower rates than observed when arising from heme-catalyzed electron transfer to dioxygen. In the presence of subsaturating concentrations of L-arginine or of cofactor BH_4, full-length NOS isoforms reduce molecular oxygen to superoxide [3, 5–7, 20, 21]. Moreover, the mechanism and enzyme site responsible for $O_2^{\bullet -}$ formation as well as the respective contribution of the reductase or oxygenase domain can vary among the three isoforms [53–56]. The physiological relevance of $O_2^{\bullet -}$ generation catalyzed by NOSs is still a matter of debate but it is now clearly established that $O_2^{\bullet -}$ and NO synthesis can occur simultaneously within NOS and that BH_4 can regulate their relative levels [54, 55].

Finally, in the presence of H_2O_2, the full-length NOSs and the dimeric NOS II oxygenase domain do not catalyze NO formation from L-arginine. However, they catalyze the oxidation of NOHA by H_2O_2 [57–59] (eq. e, fig. 2). The mechanism of this recently described reaction is not yet fully understood because it generates a new metabolite, N^{δ}-cyano-ornithine, beside L-citrulline and NOs (eq. e, fig. 2) [59].

Regulation of NOSs

In certain mammalian pathologies, NO is either under- or overproduced [3, 5–7, 9]. The first situation is found in diseases such as hypertension, atherosclerosis, and restenosis. The use of various NO donors can partially palliate NO underproductions. In contrast, it is now clearly established that a number of disease states arise from inappropriate overproduction of NO, and include rheumatic diseases, septic shock, diabetes mellitus, and cerebral ischemia [3, 5–7, 11–13]. A number of strategies have emerged to provide rational control of NO levels. Isoform-selective inhibitors will certainly be essential in any therapeutic application, particularly for

the treatment of chronic disorders. Most efforts have been directed toward selective inhibition of the inducible NOS II that could be used to treat endotoxic shock and clinical problems related to abnormally high levels of NO. As described above, since there are several important differences among the isoforms, isoform-selective inhibition should be, at least in principle, possible [11–14, 52].

Regulation of NOS activity at the transcriptional level and by post-transcriptional modifications (phosphorylation, palmitoylation), as well as prevention of enzyme induction [60, 61] are described in other reviews in this issue. We will focus on the effects of cofactor (BH_4 and L-arginine) depletion or coenzyme (CaM) inhibition. The flavoprotein inhibitor diphenyleneiodonium inhibits all NOS isoforms [62] but also inhibits any flavin-dependent electron transfer. The formation of products (NO, $O_2^{\bullet -}$, and peroxynitrite) is another possible way to regulate NOS activity and the potential of NO to inhibit NOSs is still an open question. The formation of a nitrosyl complex of NOS during turnover is clearly observed and represents about 80% of the NOS content [63]. However, this complex readily turns back to native NOS Fe^{III} in the presence of O_2. Similarly, peroxynitrite inactivates NOS II through changes in the heme environment [64]. These potential modes of inhibition seem unlikely to be of pharmacological interest.

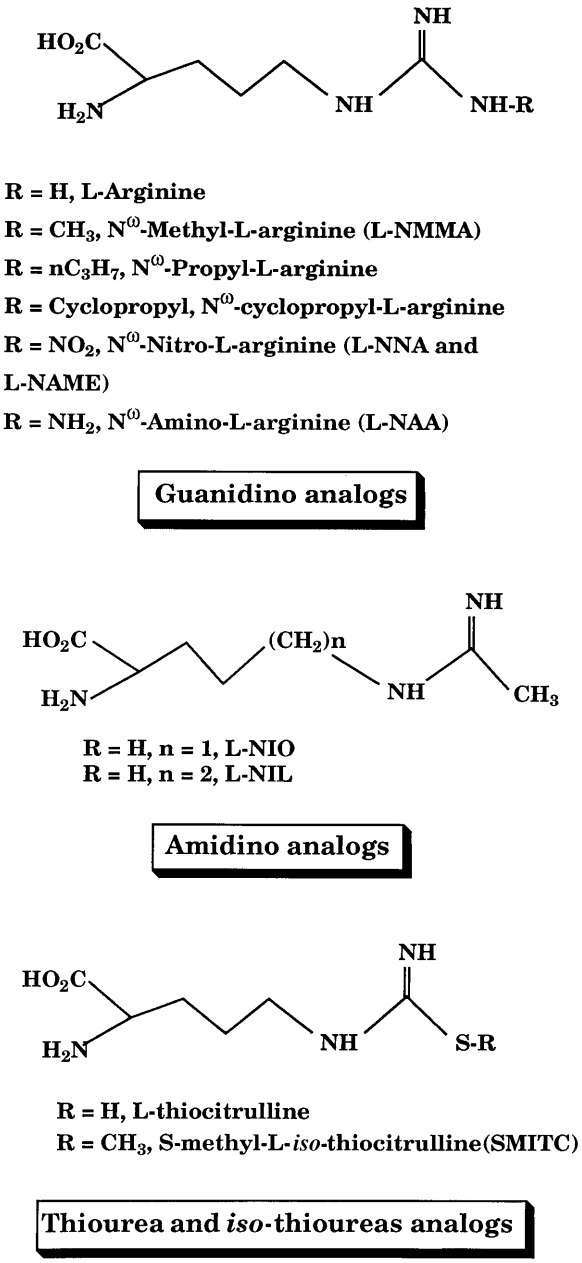

Figure 5. Structure of some L-arginine analogs, inhibitors of NOSs.

Regulation by coenzyme CaM and cofactor BH_4

Ca^{2+}/CaM antagonists

CaM regulates electron transfer from the NADPH to the heme by acting as a switch between the reductase and oxygenase domains and by stimulating electron transfer into the reductase domain itself [26, 27]. Distinction between constitutive and inducible NOS lies in their differences toward CaM binding: NOS I and NOS III bind CaM in a reversible Ca^{2+}-dependent manner. In contrast, NOS II binds CaM so tightly that its activity does not seem susceptible to control by transient variations in Ca^{2+} concentrations [11–14]. Constitutive NOS isoforms that show a typical type of CaM interaction are inhibited by Ca^{2+} chelators (EDTA, EGTA) and CaM antagonists (chlorpromazine, W7, or tamoxifen) [3, 5–7, 11–14, 65]. Due to the involvement of CaM in many physiological processes, inhibition of NOSs through CaM antagonists should be fraught with selectivity problems. However, this view is beginning to change. The interaction of CaM with NOSs appears to be unique and the design of agents that could selectively interfere with the CaM/NOS complex formation seems possible. Significant progress has been made on CaM-NOS interactions using peptides derived from the CaM-binding site [66, 67].

BH_4 biosynthesis and binding site

BH_4 binding or BH_4 biosynthesis are potentially targets for drug design because of the key role for BH_4 in dimer formation from monomers, stability of dimeric NOSs, and NO formation [20, 21, 31–35]. This type of indirect inhibition uses inhibitors of BH_4 synthesis such as 2,4-diamino-6-hydroxypyrimidine, an inhibitor of the enzyme GTP cyclohydrolase-I, and methotrexate, an inhibitor of dihydropteridine reductase [3, 5–7, 11–14]. However, interference with other BH_4-dependent en-

zymes such as phenylalanine or tyrosine hydroxylases illustrates the potential problems of nonspecific effects of these compounds. Recent crystallographic data show that dimerization results in strong modifications of the monomeric protein, including interactions between BH_4 and the heme carboxylate group, the α-NH_2 group of L-arginine, and residues involved in the dimer interface [45] (fig. 4). Better knowledge of the BH_4 binding site should allow the design of more selective pterin antagonists.

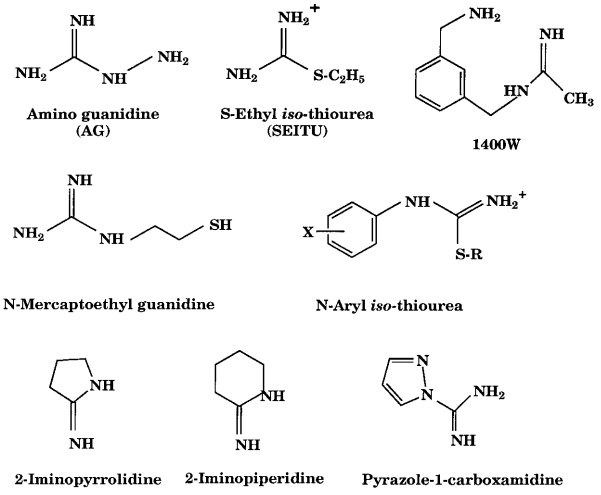

Figure 6. Structure of some nonamino acid inhibitors of NOSs.

Active-site inhibitors

Active-site inhibitors should be the most suitable tools to achieve selective inhibition of NOS and could include (i) interaction at the L-arginine binding site with competitive inhibitors, (ii) interaction at the active site with mechanism-based inhibitors, and (iii) ligands directed toward the heme.

Arginine analogs

Studies of NO synthesis have been facilitated by the early identification of N^{ω}-methyl-L-arginine (L-NMMA) as a competitive inhibitor of NO formation with a K_i in the 1–10 μM range, but with poor isoform selectivity [68]. Since this initial finding, many reports have used other simple N^{ω}-derivatives of L-arginine, or structurally related compounds to inhibit the NOSs [3, 5–7, 52] (fig. 5). N^{ω}-Nitro-L-arginine, its methyl ester, and N^{ω}-amino-L-arginine are relatively selective for the constitutive isoforms [3, 5–7, 52]. Several other alkyl analogs have been synthesized and studied. N^{ω}-Cyclopropyl-L-arginine displays modest selectivity for the constitutive isoforms whereas N^{ω}-propyl-L-arginine is a potent and selective inhibitor of the neuronal isoform [69, 70]. (Iminoethyl)-L-ornithine (L-NIO) which differs from L-arginine by the presence of an amidino group in place of a guanidino group (fig. 5), is a potent but nonselective inhibitor of NOSs. Its higher homolog, (iminoethyl)-L-lysine (L-NIL), selectively inhibits the inducible isoform, suggesting that limited changes in structure have remarkable effects on isoform selectivity [71, 72].

Recently, L-thiocitrulline and S-alkyl-L-thiocitrulline have been synthesized and evaluated [73, 74] (fig. 5). They are the most potent competitive inhibitors of both classes of NOS, with a good selectivity toward NOS I in vitro. S-Methyl-L-thiocitrulline is a potent, reversible, slow-binding inhibitor of NOS I, but seems to be less potent when tested in a cell-based enzyme assay. Higher homologs, *homo*-L-thiocitrulline and S-methyl-*homo*-L-thiocitrulline, display less efficiency and selectivity [75]. For L-thiocitrulline, the sulfur atom of the thiourea group interacts with the heme iron of NOS [45, 73]. In addition, L-thiocitrulline and N^{ω}-nitro-L-arginine specifically inhibit electron transfer from the reductase domain to the heme, inhibit dimerization of NOS II [76, 77] and suggest novel mechanisms of NOS inhibition.

Nonamino acid inhibitors

Recently, simple nonamino acid compounds have been found to be very potent inhibitors of NOSs (fig. 6). Aminoguanidine is a potent and selective mechanism-based inhibitor of NOS II [5–7, 52, 78]. It is also selective for NOS II in vivo [78]. Simple S-alkyl-*iso*-thioureas and bis-*iso*-thioureas appear to be the most potent NOS inhibitors known to date. For example, S-ethyl-*iso*-thiourea (SEITU) inhibits human NOS I, II, and III with K_i values of 29, 19, and 39 nM respectively [79] and selectivity toward NOS II can be achieved by changing the substituent. Little is known about the mechanism of action of these *iso*-thioureas. SEITU interacts with the active site of NOS II to give a type-I spectrum, as observed with L-arginine [77], and it is hypothesized that SEITU binds at the guanidine portion of the L-arginine site. Mercaptoalkyl-guanidines and substituted N-aryl-*iso*-thioureas are also potent inhibitors of the three NOS isoforms, some of them exhibiting high selectivity for the neuronal isoform versus the inducible and endothelial isoforms [80, 81]. Pharmacological use of these *iso*-thioureas should however be limited by their low tissue uptake, in vivo instability, and toxicity. Extension of these studies led to the discovery of N-(3-(aminomethyl)benzyl) acetamidine (1400W, fig. 6), a slow, tight-binding and

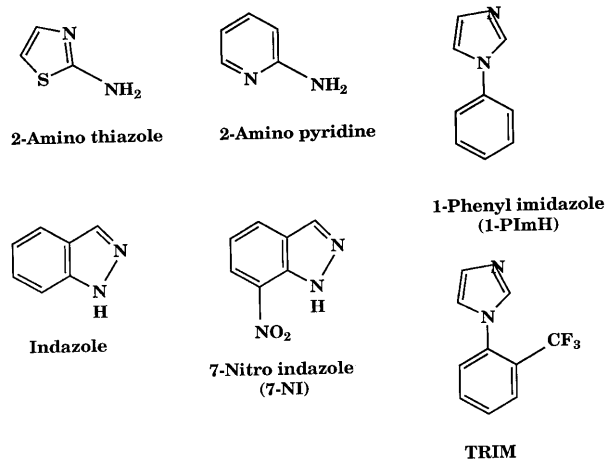

Figure 7. Some heme ligands used as inhibitors of NOSs.

highly selective inhibitor of inducible NOS, acting in vivo and in vitro [82]. In in vitro experiments, 1400W inhibits recombinant NOS II 5000-fold more efficiently than NOS III and NOS II 200-fold more efficiently than NOS I [82]. Removal of a single methylene bridge between the amidine nitrogen and the phenyl ring of 1400W gives N-(3-(aminomethyl) phenyl) acetamidine which displays high selectivity toward the neuronal isoform, indicating again that modest modification of the structure of an inhibitor can strongly alter its selectivity [83]. Finally, simple alkyl amidines such as 2-iminopiperidines, 2-iminopyrrolidines and pyrazole-1-carboxamidine are potent inhibitors of the inducible isoform [84–87] (fig. 6).

Heme ligands

NOS isoforms contain a cytochrome-P450-type heme and a general approach to inhibit this class of enzymes would be to create compounds that could be ligands of the heme. Destruction of the heme after binding is also an approach toward irreversible inhibition [reviewed in ref. 48]. The inhibitory properties of imidazole derivatives such as 1- and 2-phenyl imidazoles [88], 1-(2-trifluoromethyl phenyl) imidazole [89], the antimycotic agents miconazole [90], or heterocyclic such as 2-amino pyridines and 2-amino thiazoles may rely on their ability to coordinate with heme [41] (fig. 7). However, these imidazole derivatives inhibit NOSs with diverse efficiencies and selectivities [41, 88, 89]. Indazole and other indazole analogs such as 7-nitroindazole (7-NI) have been described as good NOS inhibitors, with good selectivity of 7-NI toward NOS I [3, 5–7, 91]. Inhibitor design based on heme ligation is likely to meet with some success, although the path toward the design of selective inhibitors based on this strategy is presently not clear.

Attempts have been made to rank the potency of these inhibitors (table 2), but comparisons must take account of differences in NOS preparations or conditions of the experiments. In in vivo studies or with cell cultures, transport or uptake of the inhibitors into cells can strongly modify these values and isoform selectivity.

Cross-regulation between arginases and NOSs

Particular interest will be directed toward the regulation of NOS activity by modulation of substrate availability in the presence of arginases. The concentrations of L-arginine measured in tissues and cells are in the 100–250 μM range [1–3], far exceeding the K_m values reported for NOSs (table 1). However, subsaturating concentrations of L-arginine result in low NO formation but high $O_2^{\bullet -}$ generation [3, 5–7, 11, 12, 20, 21]. The relative expression levels of arginases and of proteins involved in L-arginine synthesis (citrulline-arginine recycling enzymes) and transport across menbranes thus appear as major determinants controlling final NO output (fig. 1).

The arginases

Arginase (L-arginine-urea hydrolase) is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to urea and L-ornithine [4, 92] (fig. 1). Recent studies have established the presence of at least two distinct arginase genes coding for immunologically distinct isoforms [93]. One of them (A1) is strongly expressed in the liver where it functions as a key part of

Table 2. Indicative inhibitory constants (K_i) of some L-arginine analogs, nonamino acid compounds and heme ligands on NOS activity. See figures 5–7 for abbreviations.

Compound	NOS I	NOS III	NOS II
L-NMMA	0.2 μM	0.4 μM	6 μM
L-NNA	15 nM	40 nM	4.5 nM
L-NAA	0.2 μM	<0.1 μM	1.7 μM
L-NIO	3.9 μM	0.2μM	2.2 μM
L-NIL	92 μM	n.d.	3.3 μM
L-Thiocitrulline	60 nM	n.d.	3.6 μM
SMITC	5 nM	11 nM	40 nM
AG	830 μM	>8 mM	16 μM
SEITU	29 nM	39 nM	19 nM
1400W	0.14 μM	75 μM	2 μM
1-PimH	38 μM	50 μM	0.7 μM
7-NI	0.16 μM	0.8 μM	1.6 μM
TRIM (IC_{50})	28 μM	1.1 mM	27 μM

n.d.: not determined.

the urea cycle. AII is found in mitochondria of extrahepatic tissues and cells such as the red blood cell, the lactating mammary gland, the kidney, and macrophages [reviewed in refs. 4, 94–96]. The extrahepatic arginase (AII) subserves a number of as yet not well defined roles, including participation in polyamine synthesis via the cytosolic enzyme ornithine decarboxylase, and formation of proline, creatine, glutamate, agmatine, and γ-amino-butyric acid [reviewed in ref. 4]. Arginases AI and AII appear to be expressed constitutively in murine macrophages, although different stimuli may modulate the level of expression of these isoforms (see below). AI and AII contribute to immune system function since L-ornithine is a key precursor for polyamines involved in cell replication. Ornithine/urea production markedly increases during tumor growth whereas arginase activity decreases during tumor rejection [97]. Recently, it was suggested that AI and AII could be involved in the regulation of several cytostatic and cytotoxic actions of activated macrophages mediated by NO synthesis. In these cases, modulation of local L-arginine concentrations by arginases could regulate high NO production [3, 5–7, 96–99].

Substantial amounts of NOHA are liberated from the active site of inducible NOS [46, 47, 50] and it has been demonstrated that NOHA is a potent inhibitor of liver arginase [100, 101]. NOHA may thus act as an endogenous arginase inhibitor in NO-producing cells, as observed in endothelial cells or alveolar macrophages [47, 102, 103]. However, NOHA is a substrate for NOSs and is useless as a pharmacological tool to elucidate interactions between the arginases and NOS pathways. We recently synthesized a new analog of NOHA, N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) and showed that it is a potent competitive inhibitor of rat liver arginase [104] without being a substrate for NOSs [50]. Inhibition of arginase by nor-NOHA could thus increase the availability of L-arginine for NO biosynthesis in cells possessing an active NOS. nor-NOHA could be a selective tool allowing detailed studies of the interactions, between NOSs and arginases [47, 100–103, 105].

In some cases, NOS II and arginases I or II seem to be co-induced but in other cases, both types of enzyme are submitted to opposite or reciprocal regulation. Inducers can be tentatively classified into three classes: (i) mediators which induce both NOS II and arginases; (ii) mediators which exert similar or opposite effects on arginases and NOS II, depending on the cellular system; (iii) mediators which exert opposite effects on arginases and NOS II expression.

Mediators which induce both NOS II and arginases

Lipopolysaccharides (LPSs) seem to be the only class of molecules which are able to activate arginases and NOS II genes.

LPSs in vivo and in vitro induce expression of NOS II and AI and/or AII. In vivo, LPSs increase mRNA of arginases and NOS II in lung, heart, spleen, and liver of rats as well as other enzymes able to recycle L-citrulline to L-arginine (arginino succinate synthase and arginino succinate lyase) [106]. In vitro, LPSs induce arginases and NOS II in various systems including rat aortic endothelial cells [47], rat [107] and mouse peritoneal macrophages [108], the mouse macrophage cell line RAW 264.7 [109], and mouse bone-marrow-derived macrophages [110].

Although arginases and NOS II genes are both activated by LPS, their modulation can differ at the kinetic level, and be influenced by other mediators. For example, kinetic modulation was observed [106] in vivo in rats where NOS II mRNA in the spleen reached a plateau 5–6 h after intraperitoneal LPS injection, decreased thereafter and returned to a hardly detectable level at 24 h. In parallel, arginino succinate synthase mRNA increased up to 12 h and arginino succinate lyase mRNA increased continuously up to 24 h [106], probably resulting in increased L-arginine availability and compensating for the decrease in NOS II efficiency. In vitro, rat peritoneal macrophages were cultured in the presence of LPS, and NOS II mRNA appeared 2 h after LPS addition and increased up to 12 h. On the other hand, AI mRNA began to increase only after 4 h (with a lag) and reached a maximum at 12 h [106]. Unexpectedly, because AI has been thought to be expressed almost exclusively in the liver, immunoblots showed that NOS II and AI proteins were produced in macrophages [106]. The RAW264.7 NOS II level was also observed to reach a plateau after 24 h whereas the AII level increased up to 48 h [109]. Thus, there is no true co-induction of arginases and NOS II by LPS. Arginase induction appeared delayed compared to NOS II, perhaps to limit the duration of NO production. One can also mention that molecules such as pyrrolidone dithiocarbamate which prevents translocation of NF-κB, an essential transcription factor for NOS II induction [3, 5–7], are ineffective in AII induction [109].

Mediators which can exert similar or opposite effects on arginases and NOS II expression

Induction of NOS II and generation of NO in pancreatic islet β-cells may mediate interleukin (IL)-1-induced dysfunction leading to insulin-dependent diabetes mellitus [3, 5–7]. Cytosol analysis from the rat insulinoma-derived cell line RINm5F treated with IL-1β has shown that IL-1β increased NOS II and significantly decreased arginase expresssion, thereby increasing the availability of L-arginine for NO production and resulting in a long lasting cytotoxic effect of NO [111]. In contrast, when mesangial cells were stimulated with IL-1, AI, AII and

NOS II were up-regulated, IL-1 increasing arginase activity by 60% [112].

Oxygen tension can exert complex effects. Hyperoxia (100% O_2) increases AI and AII in rat lung, without affecting NOS II expression [113]. In contrast, hypoxia or anoxia increases AI and NOS II expression [114] but decreases AII mRNA expression in rat and mouse peritoneal macrophages, while L-arginine transporter mCAT-2 expression is increased more than twofold [108]. Arginases AI and AII as well as NOS II and the mCAT-2 L-arginine transporter are thus O_2-regulated genes.

Mediators which exert opposite effects on arginases and NOS II expression

As a general rule, cytokines inducing NOS II do not induce arginases and vice versa. For example, cytokines such as interferon-γ produced by the Th1 subpopulation of CD4+ T cell, induce NOS II in mouse bone-marrow-derived macrophages whereas Th2-derived cytokines such as IL-4, IL-10, and IL-13 are potent inducers of arginases [115]. Exposure of macrophage cultures to inducers of NO synthase exhausts their ability to respond subsequently to inducers of arginases. Conversely, exposure of the cells to inducers of arginase exhausts their ability to respond to inducers of NOS II. Inhibition of NOS II induction by IL-4 in murine macrophages and endothelial cells has been explained by an increased rate of NOS II mRNA degradation [116] whereas IL-10 could prevent synthesis of tumor necrosis factor-α, a cytokine inducing NOS II [117]. Arginase levels induced by Th2 cells far exceeded those inducible by the individual cytokines derived from the Th2 subpopulation. Antibody-blocking experiments revealed strong synergistic effects between IL-4/IL-13 and IL-10 sufficient to account for the high arginase activity induced by the Th2 cells [118]. During immune reactions, the NOS II and arginase enzymes appear to define two alternate macrophage functional states induced by Th1- and Th2-derived cytokines, respectively [118].

Glucocorticoids are known to inhibit NOS II induction [3, 5–7, 11–14, 60]. In contrast, AI is induced by glucocorticoids in a delayed manner. Induction of arginase mRNA by dexamethasone is preceded by an increase in C/EBP binding activity which follows an increase in C/EBP beta mRNA [107, 119]. In vivo in rats, steroids increase urea cycle enzyme mRNA levels and this effect is abolished by growth hormone [120]. Transforming growth factor (TGF)-β has been shown to down-regulate NOS II expression, probably like IL-4, by increasing the rate of NOS II mRNA degradation. TGF-β up-regulates arginase activity in rat peritoneal macrophages and hence might limit macrophage-dependent cytostasis [121]. These results are consistent with competition of both enzymes for L-arginine and a reciprocal inhibition in their induction.

Concluding remarks

Many results are now consistent with the conclusion that the pathway used by macrophages to metabolize L-arginine can influence the type of host immune responses against pathogens, parasites, or tumors. The co-induction of extrahepatic arginase and NOS by the same stimuli [106–109], the induction of arginase by suppressors of NOS [110], and the identification of compounds that co-regulate the expression of both NOS and arginases [110–115], support the hypothesis that arginase may be essential in the regulation of NOS activity by modulating local L-arginine concentrations [97–109, 121].

Arginase I, previously thought to be restricted to the liver, accounts for high arginase activity at inflammatory sites where it may limit high-output NO production and generate (along with AII) polyamines and proline involved in tissue repair and cell proliferation. Contrasting temporal expression of the NOS II and arginase pathways has been observed in inflammation, NOS II being observed at the early stages and the arginases at a later stage. IL-4, a mediator generated during inflammation, may play a major role in the switch from high NO to low NO formation and increase L-ornithine and polyamine synthesis during tissue repair. Finally, inhibition of proliferation of a human tumor cell line by NOHA and NO seems to be related to distinct mechanism, at sequential steps of the arginine-polyamine pathway. NOHA inhibits Caco-2 tumor cell proliferation likely by inhibiting arginase activity, whereas NO causes cytostasis by mechanisms that might involve inhibition of ornithine decarboxylase [122].

A recent study demonstrates that extracellularly applied arginase can inhibit neuronal apoptosis induced by multiple stimuli [123]. The protective effects of arginase could not be reproduced by an array of NOS inhibitors but rather seemed to depend on depletion of L-arginine, resulting in protein synthesis inhibition. This identifies amino acid depletion as a novel biological strategy to prevent pathological neuronal apoptosis and suggests that additional insight into the localization and regulation of arginases could elucidate novel approaches to regulate cell death and NO synthesis [123]. L-Arginine enzymes, capable of simultaneously inhibiting protein synthesis and NO generation, could be therapeutic targets for acute neurological diseases.

Conclusion

Ten years ago, the free radical NO was only considered as a highly reactive and toxic compound and it was hardly conceivable that it might serve as a beneficial mediator in biological processes. This remains true as long as it is delivered at excessive rates or excessive concentrations. Since the identification of endothelium-derived relaxing factor with NO, an increasing number of studies have clarified its key roles in physiological and pathophysiological processes in mammals. Further studies have elucidated its roles in other species such as fishes, insects, bacteria, and, very recently, plants, where it serves either as a neuromediator or as an element of the response against pathogens. This resulted in its designation as 'molecule of the year' by *Nature* in 1992 and by the award of the Nobel Prize for Medicine in 1998 to Drs. Murad, Furchgott, and Ignarro, three of the earliest investigators of the L-arginine/NO pathway.

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