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INHIBITION OF CONSTITUTIVE AND INDUCIBLE NITRIC OXIDE SYNTHASE: Potential Selective Inhibition

J. M. Fukuto and G. Chaudhuri
Department of Pharmacology and Obstetrics & Gynecology, UCLA School of Medicine, Center for Health Sciences, Los Angeles, California 90024–1735

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ABSTRACT

Nitric oxide (NO) is a molecule that has been shown to be involved in a diverse array of physiological events. A variety of disease states and disorders are, in fact, due to either an over- or an underproduction of NO. As a result of the ubiquity and diversity of NO-mediated phenomenon, pharmacological manipulation is difficult. NO biosynthesis is the result of an oxidation of a terminal nitrogen on the amino acid arginine by a class of enzymes generally referred to as the nitric oxide synthases (NOSs). Since the various isoforms of NOS are distributed in cells and tissues according to their function, there is the possibility that manipulation of NO levels can be accomplished by designing specific pharmacological agents targeted at a single NOS isoform. Thus, this review discusses general inhibition of the NOSs by a variety of agents and then focuses on the possibility of developing agents for specific isoform inhibition.

INTRODUCTION

Nitric oxide (NO) is a simple diatomic molecule that was relatively obscure to most physiologists and pharmacologists before about 1988. However, the

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original discoveries of NO biosynthesis in a variety of mammalian cells (1–5) prompted numerous studies that have implicated a role for NO in a wide array of important physiological events (for recent reviews on the physiological aspects of NO, see 6 or 7). For example, NO has been found to play a role in the vascular system, in the central and peripheral nervous systems, and in immune system response. The physiology of NO remains a topic of extreme interest as more roles for it will surely be discovered and elucidated in the future. The involvement of NO in these vital functions makes it a pharmacological target for the treatment of a variety of disorders or disease states ranging from coronary artery disease to endotoxin shock. However, the general ubiquity of NO, and the fact that it plays a primary role in so many different and seemingly unrelated events, makes its specific pharmacological and/or therapeutic manipulation a challenging task (for example, see 8). That is, nonspecific or global changes in NO levels may be therapeutic with respect to one function and yet may be devastating to other unrelated NO functions. Therefore, if manipulation of NO levels is to be a realistic therapeutic goal, fairly specific agents need to be developed for altering NO levels in certain desired tissues or cells.

NO is biosynthesized in mammalian cells through the actions of a class of enzymes generally referred to as the nitric oxide synthases (NOSs). A variety of isoforms exist that differ primarily in their biophysical properties and mechanisms of physiological regulation. However, they all share similar, if not identical, cofactor and prosthetic group requirements, and they all utilize the amino acid arginine as the substrate for NO generation. The various NOS isoforms are, for the most part, distributed in cells and tissues according to their apparent function. Thus, one obvious way to pharmacologically manipulate physiological NO levels in specific tissues or cells is to develop specific inhibitors of that particular NOS isoform. Thus, the purpose of this review is to discuss general inhibition of the NOSs by a variety of agents and then focus on the possibility of developing agents for specific isoform inhibition. However, before addressing these topics, we briefly review some of the physiology and enzymology of NO biosynthesis in order to provide a basis for further discussions.

THE PHYSIOLOGY OF NO

As previously stated, the physiology of NO is extremely diverse. In this section, some of the more well-characterized aspects of NO function will be mentioned in order to provide an appreciation for the therapeutic potential for NO manipulation, as well as to emphasize the problems associated with a species that has so many different and unrelated roles.

NO as Endothelium-Derived Relaxing Factor (EDRF)

In 1980, it was reported that a substance released from endothelial cells in response to acetylcholine exposure was capable of causing relaxation of smooth muscle tissue (9). At that time, this substance was referred to as endothelium-derived relaxing factor (EDRF) since its chemical identity was unknown. After this initial discovery, a number of studies focused on the characterization and identification of EDRF. It was found to be an unstable and transient species under normal physiological conditions (10) which elicited smooth muscle relaxation by raising intracellular cGMP levels through the activation of guanylate cyclase (11–13). Further studies indicated that EDRF could be inactivated by hemoglobin, methylene blue (14), and superoxide (O_2^-) (15, 16). Then, after original proposals by Ignarro and coworkers (17) and Furchgott (18), NO was identified as EDRF by several groups (1, 2). Although it is now fairly well accepted that EDRF is NO, some reports dispute that EDRF is purely NO (19–26). It has also been proposed that other species that either bind NO or are NO derivatives may be responsible for some of the biological properties of EDRF (27–30). Regardless, it is certain that NO is involved in EDRF-elicited vasorelaxation, and that EDRF is either NO or a molecule capable of releasing NO.

NO and the Vascular System

The identification of N-methyl-L-arginine, an inhibitor of NO biosynthesis (31–33), provided an important tool to investigate the relevance of NO in vascular physiology. N-methyl-L-arginine is a potent vasoconstrictor in vitro as well as in vivo (34, 35). This vasoconstrictor action is seen only in the presence of endothelium, and is due to inhibition of endothelial-cell NOS (discussed in detail later) (31–33). These findings led to the conclusion that there is physiologic, nitric-oxide dependent vasodilator tone that is essential for the regulation of blood flow and pressure (35). This release of NO by endothelial cells appears to be shear-stress and tone dependent (36–38), and this tone-dependent NO release may constitute a local vascular reflex mechanism to sustain regional blood flow into vascular beds in the presence of intense sympathetic vasoconstriction (37). It is possible that a dysfunctional NO-synthetic mechanism could lead to the pathogenesis of some forms of hypertension. Such a view is supported by the observation that N-methyl-L-arginine produces a hypertensive response in animals (31, 32, 34) and causes vasoconstriction of the forearm arterial circulation in humans (36), and that endothelium-mediated vasodilation is impaired in patients with essential hypertension (39).

As mentioned previously, the original work by Furchgott & Zawadzki (9) utilized acetylcholine to elicit EDRF release from endothelial cells. Clearly,

this phenomenon has little or no physiological relevance, since acetylcholine does not circulate through the vascular system. However, NO appears to play a vital role in the vascular system as an endogenous vasorelaxant, since its release can be elicited by a variety of other, more physiologically relevant agents or phenomenon—such as bradykinin or shear stress (40, 41). In the vasculature, NO release from the endothelial cells appears to be primarily a result of an influx of intracellular Ca^{2+} levels. Thus, any agent or stimuli capable of eliciting an influx of Ca^{2+} into the cell (e.g. acetylcholine, bradykinin, electrical current, shear force) can cause an activation of NO biosynthesis. The NO then can freely diffuse into the neighboring smooth muscle cells, where it activates the enzyme guanylate cyclase, which converts GTP to cGMP. Relaxation of smooth muscle is therefore a result of elevated cGMP levels. Thus, NO is utilized in the vasculature as a messenger in the cGMP signal-transduction pathway.

NO and Platelets

NO is also capable of inhibiting platelet aggregation and adhesion (for example 42–44a). Thus, NO that diffuses from endothelial cells luminally may function primarily to inhibit the adhesion of platelets to endothelial cells (44). The physiological role of NO in inhibiting platelet aggregation in vivo may be less important compared to prostacyclin, because the circulating red blood cells that contain hemoglobin can inactivate NO. It appears that the constitutive NO synthase regulates platelet adhesion to endothelial cells (44a).

Stimulation of SGHEC-7 (primary human umbilical vein endothelial cells [HUVEC] transfected with EcoR-1 linearized plasmid pSV 3neo) cells (45), which retain a number of differentiated endothelial cell functions by cytokines (IL-Iβ and TNFα), increases their adhesive properties to platelets and at the same time causes them to express the inducible NO synthase. This increased adhesion of platelets to the cytokine-stimulated HUVEC is partly attributable to cytokine-induced expression of adhesion molecules in the endothelium (46) and could be enhanced by dexamethasone, an inhibitor of the expression of the inducible NO synthase (47), or by L-NMA, a direct inhibitor of the enzyme (31, 32, 48). Thus, the concomitant expression of the inducible NO synthase may modulate the pro-adhesive and thrombogenic action of IL-Iβ and TNFα (46). These findings may help explain the observation that glucocorticoids may cause vascular occlusion when used to control inflammation of the vessel wall (49).

The anti-aggregatory and anti-adhesive effects of NO are a result of increased cGMP within the platelets. Presumably, the increased cGMP causes an extrusion of intracellular Ca^{2+} from the platelets, which leads to an inhibition of adhesion and aggregation (50). Platelets themselves have been reported to

be capable of synthesizing NO, and thus they may regulate platelet aggregation and adhesion by a negative-feedback mechanism (47).

NO in the Immune System

NO appears to be widely utilized in the immune system as a cytotoxic or cytostatic agent. For example, when macrophages are exposed to infectious agents, there is a dramatic increase in nitrogen oxide formation as measured as nitrite (NO_2^-) and nitrate (NO_3^-) production (51). The precursor of NO_2^- and NO_3^- was shown to be NO produced from the oxidation of a terminal guanidinium nitrogen on the amino acid arginine (3, 4, 52). Thus, NO synthesis by activated macrophages represents an immune system response to infection (53, 54) or tumor formation (5, 55). When cultured in vitro, peritoneal macrophages obtained from mice previously inoculated with Bacillus Calmette Guerin (BCG) release NO, which is cytostatic and/or cytolytic for tumor cells (56, 57). This in vitro cytostatic and/or cytolytic activity is also observed after activation of the macrophages by cytokines (58, 59). NO produced by the activated macrophages is responsible for this cytostatic and/or cytolytic activity (4, 5, 60). Interferon-γ (IFN-γ) is important for the priming of macrophages, and tumor necrosis factor–α (TNF-α) or some other cytokine or bacterial lipopolysaccharide is necessary for full induction of activated macrophage cytotoxicity (58, 59, 61, 62). More recently, it has been demonstrated that NO plays an important role in vivo in mediating both the host resistance to a syngeneic ovarian tumor in BCG-inoculated mice and the host resistance to a xenogeneic ovarian-tumor graft (63). Human ovarian malignant tumors can also produce NO (64). BCG-induced in vivo synthesis of nitrogen oxides also offers protection against *Francisella tularensis*, and this protection is lost after inhibition of NO synthesis (65). When generated as a result of an immune system response, NO has been proposed to play a direct role as a cytotoxic chemical agent. For example, it has been proposed that NO can easily penetrate the target cells and complex essential metals to disrupt critical cell function. Binding of NO to iron-sulfur proteins has been demonstrated (66), which can result in an inhibition of mitochondrial respiration, DNA synthesis, and aconitase activity (4).

Along with NO, activated macrophages also produce significant amounts of superoxide (O_2^-). Thus, the macrophage-generated NO and O_2^- can react with each other, at a near-diffusion-controlled rate (67), to generate a highly oxidizing species, peroxynitrite ($ONOO^-$). Protonation of $ONOO^-$ gives peroxynitrous acid (ONOOH), which can then decompose to NO_3^-. The decomposition of ONOOH has been shown to produce nitrogen dioxide, NO_2, and hydroxyl radical (·OH) (68), both of which are potent oxidants and may be expected to be cytotoxic. Alternatively, it has been proposed that ONOOH may itself have cytotoxic properties without going through NO_2 or ·OH for-

mation (69). Regardless, $ONOO^-$ has been demonstrated to be reactive with a variety of cellular components and may, therefore, significantly contribute to the overall cytotoxic effect of macrophage-released NO (for example, 70–73).

NO and the Central Nervous System (CNS)

An NO pathway in the central nervous system has been shown to be intimately linked with the actions of excitatory amino acids such as glutamate (73). For example, glutamate, L-aspartate, kainate, and N-methyl-D-aspartate (NMDA) cause a Ca^{2+}-dependent increase in cGMP in the CNS (for example, 74, 75). Early studies leading to the discovery of the participation of NO in this signal-transduction system demonstrated that authentic NO was capable of cerebellar guanylate-cyclase activation (76). Also, a low-molecular-weight species derived from arginine and capable of guanylate cyclase activation was present in rat forebrain (77, 78). Moreover, activation of NMDA receptors resulted in the release of a factor with striking similarity to EDRF or NO (79). The formation of NO was then demonstrated in rat forebrain (80).

It has been proposed that postsynaptic receptor activation by, for example, glutamate, results in an influx of Ca^{2+}. The increase in intracellular Ca^{2+} then stimulates NO synthesis. NO can then diffuse into neighboring cells such as the presynaptic terminal or astrocyte process to activate guanylate cyclase, which then results in the elevation of intracellular cGMP levels. The function of cGMP in the CNS is complex and includes a direct effect on ion channels, activation of protein kinases, and stimulation or inhibition of phosphodiesterase activity (81). Interestingly, the ability of NO to freely diffuse back to the presynaptic terminal after its transmitter-elicited synthesis in the postsynaptic terminal would allow it to act as a retrograde messenger. This property of NO has prompted many investigators to propose it to be the retrograde messenger required for long-term potentiation or memory formation (79, 82–86).

NO in the Peripheral Nervous System

Neurons which are neither adrenergic nor cholinergic (referred to as NANC or nonadrenergic, noncholinergic neurons) innervate a variety of smooth muscle tissues such as the gastrointestinal tract, corpus cavernosum, and esophagus. These neurons are named for what they are *not*, rather than what they are, since the identity of the neurotransmitter has yet to be determined. NANC-mediated events appear to utilize the NO-cGMP pathway, and it has even been proposed that the elusive NANC neurotransmitter is NO. However, the actual identity of the NANC neurotransmitter remains a matter of some controversy (for example, 87–90). Regardless, there appears to be little doubt that NO is a crucial mediator in NANC-mediated events.

Other Aspects of NO Physiology-Toxicology

Although the previous discussion is by no means a comprehensive listing or in-depth treatment of NO physiology, it should give an indication of the functional diversity and importance of NO. It is likely that other roles for NO in other cells and tissues will be discovered as well, adding further to the already bewildering array of NO functions. As mentioned earlier, NO can be used either in signal tranduction (i.e. NANC-mediated events, EDRF, etc) or as a cytotoxic agent in response to a challenge to the immune system. This dichotomy of function can lead to severe physiological problems in the event that NO generation (as a result of an immune system response) effects other, unrelated NO-mediated functions or vice versa. One of the primary examples of this is septic shock (see 91). An overproduction of NO as a result of exposure to bacterial endotoxins can cause extreme hypotension and/or cardiovascular collapse. A reversal of endotoxin shock can be seen with NO biosynthesis inhibitors in dogs (92) and rats (93), and has actually been used—with some success—in humans as well (94). Also, in culture, inflammatory cytokine-mediated induction of NO synthesis in microglia results in neuronal cell-death; this toxicity can be predictably exacerbated or attenuated by manipulation of NO production by the microglia (95).

In primary cortical cultures, it has been shown that NO mediates glutamate neurotoxicity, and that protection from glutamate-elicited toxicity can be attenuated in the presence of NO synthesis inhibitors (86). Also, NO normally utilized in glutamate signal transduction can become especially toxic under conditions of ischemia-reperfusion in the brain. That is, ischemia depolarizes membranes and allows an influx of Ca^{2+} into the neurons. The high levels of intracellular Ca^{2+} can then activate NO biosynthesis. It is then postulated that when the cells are reperfused with oxygen, the high levels of NO, in combination with other active oxygen species resulting from the reperfusion (i.e. O_2^-) will result in cell damage (possibly through the formation of ONOOH) (96).

Other toxicological problems associated with NO production have also been reported. NO production by macrophages can be especially toxic to pancreatic islet cells, and can thus be a cause of type 1 diabetes (97). Exposure of endothelial cells to endotoxin/interferon or tumor-necrosis factor can also cause NO-mediated cytotoxicity (98, 99). Suppression of arthritis is found when nitric oxide synthesis is inhibited (100). Nitric oxide has also been found to be able to deaminate DNA and cause genomic alterations (101). Although other instances of deleterious effects resulting from endogenous NO production have been reported, the above examples should suffice in showing how NO can have severe toxicological implications.

It should now be evident that inhibition of NO biosynthesis may have

therapeutic applications in instances where NO is mediating an unwanted toxicological event. Certainly, considering the ubiquitous physiology of NO, it would be unwise to inhibit NO biosynthesis in a global fashion. For example, general NO inhibition in the treatment of reperfusion toxicity may also result in severe and possibly fatal hypertension. Thus, a reasonable goal would be to develop specific inhibitors for NO biosynthesis occurring in specific tissues or cells. If NO were synthesized in the same manner in all cells and tissues, this would certainly be a difficult goal, since specificity at the enzyme level would be difficult or impossible. Luckily, however, NO biosynthesis differs in many cells and tissues, depending somewhat on its presumed function. These differences are manifested at the level of the enzyme protein. Therefore, any attempts to develop functionally specific inhibitors of NO biosynthesis (for example, to treat septic shock without compromising other functionally unrelated NO-mediated events), should start with an understanding of the enzymology of NO biosynthesis.

THE ENZYMOLOGY OF NOS

NO is biosynthesized in mammalian systems via the enzymatic oxidation of a terminal guanidinium nitrogen on the amino acid L-arginine (3, 4, 80, 102). The other product of this reaction is the urea equivalent amino acid, citrulline, which is formed in stoichiometric amounts (103). This reaction constitutes an overall 5-electron oxidation of the guanidinium nitrogen (Figure 1). That is, the guanidinium nitrogen atom changes its oxidation state from –III to +II in going from a guanidine to free NO (the formal oxidation state of carbon remains unchanged).

NO biosynthesis from arginine is carried out by a class of enzymes known collectively as the nitric oxide synthases (NOS). As might be expected on the basis of its diverse and ubiquitous physiology, NOS activity can be found in a wide variety of cells and tissues such as liver, brain, heart, artery, vein, adrenals,

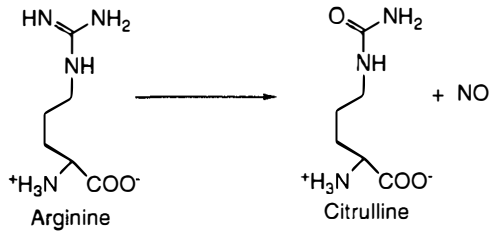


Figure 1 Conversion of arginine to citrulline and NO.

lung, and spleen; and in a variety of cells such as macrophages, endothelial cells, platelets, fibroblasts, neutrophils, astrocytes, hepatocytes, mast cells, adenocarcinoma cells, neuroblastoma, mesangial, microglial, and epithelial cells (104). This list is sure to grow as research continues. To go along with its somewhat widespread distribution, there are a variety of NOS isoforms. All of the NOS isoforms characterized thus far have common cofactor and prosthetic group requirements: NOS is an ironheme protein (105–108) which utilizes O_2 (for example, 109) and NADPH (110, 111) and requires FAD, FMN (112–115), and tetrahydrobiopterin (H_4B) (116) for activity. They also all have a strict requirement for L-arginine as D-arginine is a nonsubstrate. Since they all have common cofactors and prosthetic groups, and apparently perform the requisite oxidations via reductive activation of molecular oxygen, it would not be unreasonable to assume that they all perform similar, if not identical, chemistry. That is, the mechanism by which they all convert arginine to NO and citrulline is likely to be the same. After all, it seems unreasonable that nature would have evolved to produce multiple ways of performing what appears to be a very difficult and complex chemistry. However, along with the differences in tissue or cellular origin, the isoforms do differ significantly with regard to their biophysical properties and in their mechanisms of regulation.

NOS can be immediately divided into two distinct classes: an inducible class (iNOS) and a constitutive class (cNOS). Within each class, subclasses exist. For example, constitutive NOSs have been isolated and characterized from endothelial cells and brain, and are sometimes referred to as eNOS and nNOS, respectively. Although both subclasses are constitutive, they differ substantially in their biophysical properties (discussed later). Inducible NOS has been found in a variety of cells, and although most of the enzymological work has been performed on iNOS from macrophages, it has also been found in, for example, smooth muscle, hepatocytes, microglial cells, and neutrophils. Another designation for the various isoforms has been forwarded that classifies the isoforms according to their origin and properties (e.g. the neuronal-based isoform is referred to as type Ia, the soluble inducible isoform from macrophages is given the type II designation, and the constitutive particulate isoform from endothelial cells is referred to as type III) (117). For the sake of simplicity, only the iNOS and cNOS designations will be generally used herein, along with specific mention of the tissue or cell of origin. Although this is something of an oversimplification, it will avoid any unwarranted confusion.

NOS has been purified from a variety of sources such as rat cerebellum (soluble form) (118, 119), rat cerebellum (particulate form) (120), porcine cerebellum (121), bovine brain (122), bovine aortic endothelial cells (123), human cerebellum (124), macrophages (113, 125, 126), and rat liver, (127). Table 1 gives a listing of some of the more well-characterized NOS isoforms, and some of their physical and kinetic properties.

Table 1 Properties and characteristics of several purified NOS isoforms

Enzyme source (Ref.)	Type/regulation/dependence[a]	Physical properties	Kinetic constants
Rat cerebellum (118)	Constitutive, Ca^{2+}-CaM/NADPH	Soluble, 150 kDa (DN[b]), 200 kDa (N[c])	$K_m$arg = 1.5 μM, V_{max} = 0.96 μmol citrulline/min/mg
Rat cerebellum (119)	Constitutive/Ca^{2+}-CaM/NADPH	Soluble, 279 kDA (N), 155 kDa (DN)	$K_m$arg = 2.2 μM, V_{max} = 107 nmol citrulline/min/mg
Rat cerebellum (120)	Constitutive/Ca^{2+}-CaM/NADPH, FAD, H_4B[d]	Particulate, 150 kDa (DN)	$K_m$arg = 15.5 μM, V_{max} = 20 nmol citrulline min/mg
Bovine endothelial cells (123)	Constitutive/Ca^{2+}-CaM/NADPH, H_4B	Particulate, 135 kDa (DN)	$K_m$arg = 2.9 μM, V_{max} = 15.3 nmol citrulline/min/mg
Porcine cerebellum (121)	Constitutive/Ca^{2+}-CaM/NADPH, H_4B	Soluble, 160 kDa (DN), 200 kDa (N)	K_m not reported, S.A.[e] = 0.73 μmol citrulline/min/mg
Human cerebellum (124)	Constitutive/Ca^{2+}-CaM/NADPH, H_4B	Soluble, 160 kDa (DN), homodimer (N)	K_m not reported, S.A. = 74.3 nmol citrulline/min/mg
Bovine brain (122)	Constitutive/Ca^{2+}-CaM/NADPH, H_4B	Soluble, 150 kDa (DN), 300 kDa (N)	$K_m$arg = 2.3 μM, V_{max} = 115 nmol citrulline/min/mg
Rat macrophages (126)	Inducible/protein synthesis/NADPH, H_4B, dithiothreitol	Soluble, 150 kDa (DN), 300 kDa (N)	$K_m$arg = 32.3 μM, V_{max} = 1052 nmol citrulline min/mg
Rat macrophages (113)	Inducible/protein synthesis/NADPH, H_4B, FAD	Soluble, 130 kDa (DN), 260 kDa (N)	$K_m$arg = 16 μM, V_{max} = 26 nmol/hr
Rat macrophages (125)	Inducible/protein synthesis/NADPH, H_4B, FAD, thiol	Soluble, 125–135 kDa (DN), 250 kDa (N)	$K_m$arg = 2.8 μM, V_{max} = 1.3 μmol NO_x/min/mg
Rat liver (127)	Inducible/protein synthesis/CaM, H_4B, FAD, FMN	Soluble, 135 kDa (DN)	$K_m$arg = 11 μM, S.A. = 462 nmol citrulline/min/mg

[a] Dependence refers to factors that had to be added to purified preparations for maximum activity.
[b] DN = Denatured protein (SDS/PAGE analysis).
[c] N = Native protein (typically determined by gel-filtration analysis).
[d] H_4B = Tetrahydrobiopterin.
[e] S.A. = Specific Activity.

Regulation of NOS

As indicated in Table 1, all reported cNOS isoforms are regulated by Ca^{2+} via the Ca^{2+}-binding protein calmodulin (CaM) (118). Apparently, a Ca^{2+}-CaM complex forms as a result of Ca^{2+} influx into the cell, which then binds cNOS, with the result of activation. Thus, any event which results in the influx of Ca^{2+} into a cell containing cNOS can cause an activation of cNOS and increased NO levels. For example, acetylcholine, bradykinin, or shear stress will cause an influx of Ca^{2+} into endothelial cells, which results in NO biosynthesis. Glutamate activation of the NMDA receptor also results in an influx of Ca^{2+} and an increase in NO production in glutaminergic neurons. However, unlike the constitutive enzymes, the inducible isoforms are not regulated by Ca^{2+}. Interestingly, iNOS from macrophages does contain tightly bound CaM, which can be considered an enzyme subunit (128). That is, in cNOS, CaM binding is regulated by Ca^{2+}, whereas in macrophage iNOS, CaM is always bound regardless of Ca^{2+} levels. Another iNOS isolated from rat liver can be activated by CaM addition but is relatively insensitive to Ca^{2+} (127, 129). This is consistent with the general idea that iNOS isoforms require or contain CaM but are not regulated by Ca^{2+}. Ca^{2+}-CaM activation of cNOS has been studied spectroscopically (130). It was found that binding of the Ca^{2+}-CaM complex to cNOS allows the transfer of electrons from NADPH to the heme group to occur. Thus, with iNOS it would not be unreasonable to speculate that the tightly bound constitutive CaM allows continuous electron flow.

Phosphorylation may also play a regulatory role in cNOS. For example, cloning of complementary DNA from rat cerebellum cNOS indicated that the enzyme contains sites for phosphorylation (131). Indeed, cNOS purified from rat brain cNOS can be phosphorylated by Ca^{2+}- and CaM-dependent protein kinase II and protein kinase C (PKC) (132). In that study, phosphorylation by the Ca^{2+}- and CaM-dependent protein kinase II resulted in a marked decrease in enzyme activity, while phosphorylation by protein kinase C caused a slight increase in activity. Another study investigating the possible phosphorylation of purified rat and porcine brain cNOS by CaM kinase II, CaM kinase-Gr, PKC, and protein kinase A showed exclusive phosphorylation by protein kinase A with no consequent change in enzyme activity (133). Phosphorylation of rat brain cNOS by cAMP-dependent kinase, PKC, and Ca^{2+}- and CaM-dependent protein kinase was shown to occur at different serine sites, with a significant decrease in enzyme activity occurring after PKC activation in intact cells (134). These somewhat paradoxical reports indicate that although NOS is subject to phosphorylation, the role of phosphorylation in its regulation needs further investigation.

Unlike cNOS, iNOS appears to be regulated primarily via the induction of de novo enzyme protein synthesis. The availability of substrate or cofactors

(most notably H_4B) may also play a role in iNOS regulation in macrophages and smooth muscle (104, 135–137) as well as in cNOS (138, 139). The lack of finely tuned regulatory mechanisms for iNOS, relative to cNOS, may be due to the difference in function between the two isoforms. Because cNOS-derived NO is involved in the maintenance of vascular tone and neurotransmission, its physiological concentrations may be under stricter regulation compared to iNOS-derived NO, which is made in large quantities as a cytotoxic agent.

Biophysical Properties of NOS

As Table 1 shows, denatured forms of NOS, whether constitutive or inducible, have molecular weights in the range of 130–160 kDa. However, molecular weight analysis of the native proteins shows a molecular weight range of about 200–300 kDa. Thus, it appears that both cNOS (119) and iNOS (113, 125, 126) exist as homodimers in the native form. Detailed studies with iNOS from macrophages indicate that only the dimeric protein is active (140). That study also indicated that the monomeric enzyme retained its ability to bind CaM, FAD, and FMN but was unable to bind heme or H_4B. The active dimeric protein contained, as expected, CaM, FAD, FMN, heme, and H_4B. Also, dimerization of the subunits was promoted by heme, H_4B, and arginine. The relative activities of monomeric and dimeric forms of cNOS have not yet been determined, and although it is likely that dimeric cNOS is active, it remains to be seen if the monomeric protein has any activity. One study on cNOS indicates that nonionic detergents increase the activity of purified cNOS from rat brain, possibly by stabilizing the active dimeric protein or inhibiting dissociation of a cofactor or prosthetic group (141).

Although the majority of well-characterized NOS isoforms are cytosolic, as indicated in Table 1, membrane-bound isoforms of NOS also exist. In fact, in bovine aortic endothelial cells, the major cNOS isoform is particulate in nature (142). Association of the particulate cNOS from endothelial cells with membranes has been shown to be a result of N-terminal myristoylation (143–145). Also, along with the more well-characterized soluble isoforms from rat brain and macrophages, particulate forms of NOS have been reported from these sources as well (120, 146).

MECHANISM OF NOS-CATALYZED ARGININE OXIDATION

As a preface to discussing NOS inhibition (especially mechanism-based suicide inhibition) it is necessary to at least briefly review some of the salient mechanistic aspects of NOS catalysis. To be sure, the chemistry of the NOS-catalyzed conversion of arginine to citrulline is, as yet, incompletely under-

stood. However, based on the cofactor and prosthetic group requirements, NOS bears a striking similarity to the more well-understood cytochrome P450 system (P450). Cytochrome P450 is one of the major metabolic proteins in the liver (and other tissues), and is capable of oxidizing a variety of seemingly unreactive chemical functions (for a recent review, see 147). While NOS catalysis is carried out by a single protein, the mammalian P450 system consists of two proteins: an NADPH-binding and FAD- and FMN-containing P450 reductase protein, and a heme-containing, oxygen-activating cytochrome P450 protein. The P450 reductase protein is responsible for supplying cytochrome P450 with electrons for the heme-mediated activation of molecular oxygen. Since both NOS and P450 are hemeproteins which utilize NADPH and O_2 and require FAD and FMN, it would not be unreasonable to assume that there might be some mechanistic similarities. A generally accepted mechanistic scheme for P450-catalyzed oxidations is shown in Figure 2. In this mechanism FAD and FMN serve as an electron conduit to shuttle electrons from NADPH to the iron heme. The iron heme is thus responsible for binding and reductively activating molecular oxygen. A two-electron oxidation of the substrate then occurs via a high-oxidation state iron-oxo complex. Consistent with the idea that NOS catalysis is mechanistically analogous to P450, it has been found that arginine is initially N-hydroxylated to give, as a biosynthetic intermediate, N-hydroxy-L-arginine (148, 149). This appears to be a typical two-electron monooxygenation of the nitrogen atom of the guanidinium functionality, a process that has precedence in P450 catalysis (150). NOS oxygenase activity has been confirmed, as the oxygen atoms in both NO and citrulline originate from molecular oxygen (as opposed to water) (109). Also, further oxidation of N-hydroxy-L-arginine to citrulline and NO by NOS has been shown to involve the ironheme moiety (151) and require only one electron from NADPH (148). It should be realized that NOS catalysis differs from that of P450 in two major ways: 1. NOS requires H_4B, whereas P450 does not, and 2. the presumed product of NOS catalysis is an odd-electron free-radical species, NO, whereas typical P450-mediated oxidations are even, two-electron processes. The requirement for an odd-electron product has been reconciled by a variety of workers who have proposed that the N-hydroxy-L-arginine intermediate itself acts as a reducing agent for the enzyme, and thus only requires a single electron from NADPH for further catalysis. Alternatively, it remains possible that N-hydroxy-L-arginine is converted to citrulline and the two-electron nitrogen oxidation product, HNO, which can donate an electron back to the enzyme to give NO (152, 104). Model studies indicate that HNO can be a product of N-hydroxy-L-arginine oxidation (152, 153) and that HNO is easily converted to NO with a variety of relatively mild oxidants (154).

The requirement for H_4B by NOS is a subject of considerable interest and speculation. In other monooxygenase enzymes unrelated to NOS—such as

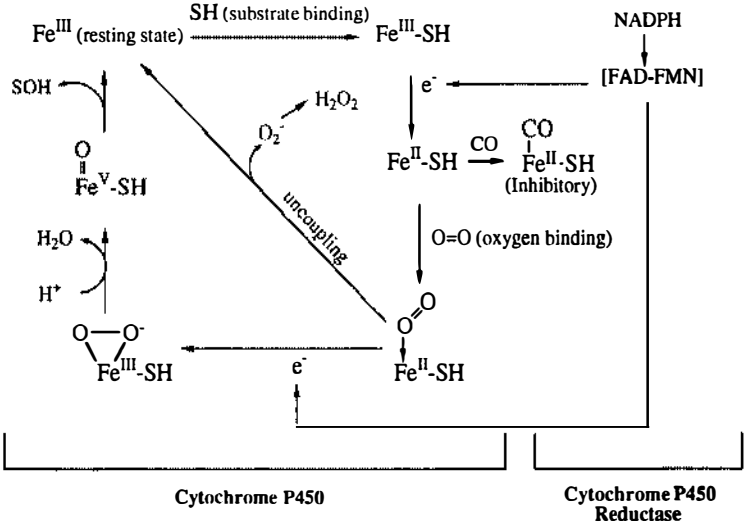


Figure 2 Mechanistic scheme for cytochrone P450 catalyzed oxidations.

phenylalanine hydroxylase or tyrosine hydroxylase—H_4B is also required for catalytic activity. It has been proposed in those systems that H_4B has a stoichiometric redox role, as it is directly involved in the activation of oxygen (155). A number of studies regarding the role of H_4B in NOS catalysis have been performed. A nonstoichiometric role for H_4B in NOS has been proposed, since citrulline formation in rat cerebellar NOS was not stoichiometric with H_4B utilization, and H_4B was apparently not recycled by NOS (as measured by coupling H_4B synthesis with phenylalanine hydroxylase activity) (156). It was suggested that H_4B was not stoichiometrically redox active and may act as an allosteric modulator or function to protect NOS by keeping a crucial catalytic component in the reduced state. Later it was shown that H_4B copurifies with both cNOS (115) and iNOS (157), and since it is possible that recycling of H_4B can occur utilizing NADPH in the NOS system (115), it is possible that redox cycling of H_4B occurs while still bound to the NOS enzyme. In a study utilizing model H_4B analogs of differing redox ability, other investigators have proposed a redox role for H_4B (157). H_4B may (among others roles) have an allosteric function, as it has been shown that H_4B promotes the formation of the active dimeric protein from its inactive monomers (140). Clearly, the actual role of H_4B in NOS catalysis is still a matter of significant uncertainty, and further studies are needed to unravel this mystery.

INHIBITION OF NOS

Considering the various mechanisms by which it is regulated and its requirement for so many cofactors and/or prosthetic groups, inhibition of NOS can be envisioned to occur in a variety of different ways. For example, inhibition of either iNOS or cNOS activity could occur by utilizing agents which compete for L-arginine, NADPH, flavin, or BH_4 binding to the enzyme. Agents which ligate the heme group of NOS might also be potent inhibitors of either isoform. Furthermore, since cNOS is regulated by intracellular Ca^{2+} levels, agents which interfere with Ca^{2+} influx or CaM binding should also inhibit enzyme activity. Inhibition of in vivo iNOS activity could be accomplished by blocking induction of de novo protein synthesis, since protein synthesis appears to be a primary mechanism of its regulation. Also, with what is known about the mechanism of NOS catalysis, mechanism-based inhibitors can be developed. Indeed, inhibition of NOS has been accomplished by a variety of these, and other, methods. Below are given examples of NOS inhibition using a variety of strategies. This listing is not intended to be comprehensive, but only to serve as representative examples of the types of NOS inhibition currently known.

Inhibition of NOS Flavoprotein Function

Both cNOS and iNOS require NADPH-dependent FAD and FMN. Therefore, it would be expected that flavoprotein inhibitors should inhibit NOS activity. Diphenyleneiodonium (DPI) and several of it analogs (see Figure 3) are known inhibitors of NADPH-flavoproteins and were found to be fairly potent inhibitors of both iNOS from macrophages and cNOS from endothelial cells (114). The inhibition of NOS was found to be time and temperature dependent, irreversible, and independent of enzyme turnover. DPI was also shown to be a potent in vivo and in vitro irreversible inhibitor of endothelium-dependent

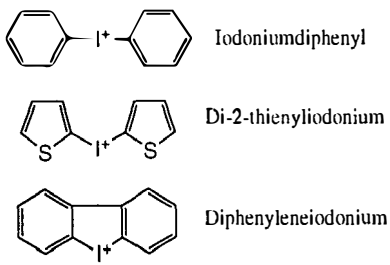
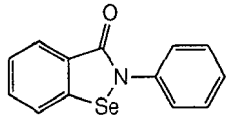


Figure 3 Structures of iodonium inhibitors of NOS.



Ebselen

Figure 4 Structure of the thiol-modifying agent, ebselen.

vasorelaxation in rat aortic rings (158) and, again, this was suggested to be a result of antagonism to the effects of FAD and NADPH.

Inhibition of Thiol Function

Inhibition of cNOS from endothelial cells by the nontoxic organoselenium compound, ebselen (see Figure 4), has been demonstrated (159). Inhibition of cNOS activity was observed in crude homogenates of bovine aortic endothelial cells. Inhibition of acetylcholine- or A23187- (a Ca^{2+} ionophore) stimulated release of NO from rabbit aortic rings was also observed. Significantly, the inhibitory action of ebselen was prevented by the addition of thiols. Thus, it was hypothesized that ebselen is capable of modifying a critical thiol on cNOS from endothelial cells by forming a selenyl-sulfide, and that exogenous thiols can protect NOS by intercepting and trapping ebselen. Interestingly, ebselen was a considerably less potent inhibitor of soluble cNOS from porcine brain or iNOS from rat spleen when compared to the endothelial cell cNOS. However, ebselen was previously found to inhibit iNOS from LPS-treated Kupfer cells in the low micromolar range in another study (160). Thus, ebselen should not be considered to be a true isoform-selective inhibitor.

Inhibition of CaM Function

As previously mentioned, cNOS is activated (and regulated) by Ca^{2+} via the reversible binding of CaM. On the other hand, iNOS is not regulated by Ca^{2+} since CaM is tightly bound and is more of an enzyme subunit. It would, therefore, be predicted that an inhibitor of CaM function would have a significant effect on cNOS activity while having little or no effect on iNOS. This concept was tested and, predictably, it was found that the CaM inhibitors calmidazolium, W-7, and fendiline were capable of inhibiting the endothelium-dependent acetylcholine-, A23187-, and ATP-stimulated relaxation of rat thoracic aorta (161). Induction of iNOS in rat aorta was then accomplished by treating endothelium-denuded aortic rings with interleukin-1B. iNOS induction and consequent increase in NO production was then demonstrated by showing that contractions to phenylephrine were inhibited. Not surprisingly, the CaM

inhibitors had little effect on restoring the contractility of the tissues with induced iNOS. (All tissues, however, responded predictably to a nonspecific NOS inhibitor). In purified systems, it was also demonstrated that CaM inhibitors were capable of inhibiting cNOS (162). These results support the general tenet that cNOS, and not iNOS, is regulated by CaM, and that cNOS activity can be inhibited by CaM inhibitors without affecting iNOS activity.

Inhibition with Heme-Binding Agents

Both iNOS and cNOS are ironheme-containing proteins. The heme prosthetic group is required for catalytic activity and is likely responsible for oxygen activation in a manner similar to that of other heme-containing monoxygenases such as cytochrome P450 (discussed earlier). The participation of the heme moiety in NOS catalysis can be inferred from studies indicating that heme-binding agents inhibit the activity of the enzyme. For example, carbon monoxide (CO) is an extremely good ligand for ferrous (Fe^{2+}) hemes and is a potent inhibitor of NOS activity (105, 107, 108). Other heme ligands such as cyanide, miconozole (107), and even NO itself (163, 164) were also capable of NOS inhibition. Inhibition of NO formation from the biosynthetic intermediate, N-hydroxy-L-arginine, can also be accomplished using CO, which suggests that the heme is required in all the catalytic steps (151). Also, imidazole and related phenylimidazoles (see Figure 5) have been found to be inhibitors of cNOS from bovine brain and GH_3 pituitary cells, presumably by ligating the heme iron and blocking oxygen activation (165). Several other antifungal

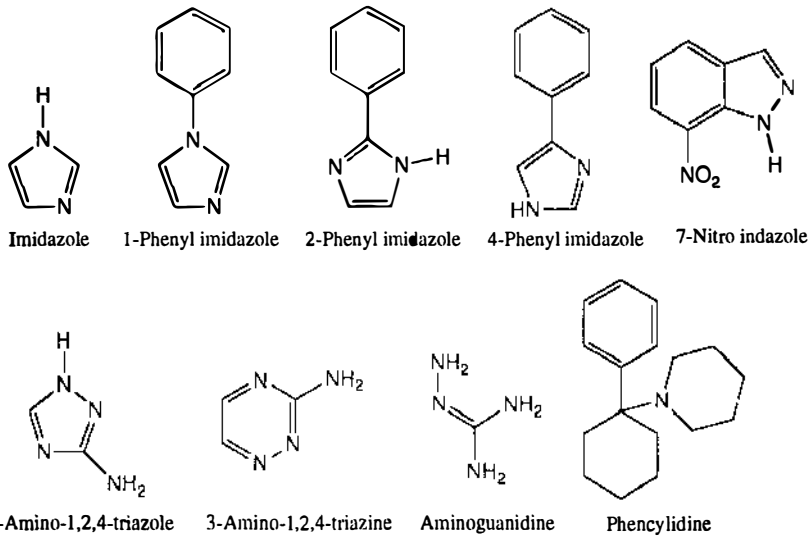


Figure 5 Structures of some nitrogen-containing inhibitors of NOS.

imidazoles were also found to inhibit cNOS by a combination of heme binding and interference with CaM function (166).

Inhibition of NOS by Inhibiting H_4B Biosynthesis

As mentioned previously, H_4B is an essential factor for both iNOS and cNOS activity. Thus, inhibition of H_4B biosynthesis should lead to a decrease in NOS activity. Consistent with this concept, it has been demonstrated that inhibition of H_4B biosynthesis can result in a loss of both iNOS and cNOS activity in a variety of systems, including endothelial cells (167, 168), cytokine-stimulated fibroblasts (169), and macrophages (170).

Inhibition of NOS by Nitrogen-Containing Compounds

A variety of amines and other nitrogen-containing compounds have been found to be inhibitors of both iNOS and cNOS activity (representative structures of some of these compounds are shown in Figure 5). Although the exact mechanism of action of many of these compounds has yet to be unequivocally elucidated, they do provide leads for the possible development of pharmacological tools. For example, 3-amino-1,2,4-triazole, a known inhibitor of catalase and peroxidase, and the related 3-amino-1,2,4-triazine are capable of inhibiting nitrite excretion and parasite killing by activated murine macrophages (171). It was suggested that the resemblance between a tautomeric form of 3-amino-1,2,4-triazine and the guanidinium functionally on arginine might be responsible for the observed inhibition. Using in vitro assays, 7-nitroindazol and related compounds have been shown to be fairly potent and specific competitive inhibitors of arginine in brain cNOS. Thus, these compounds may have future utility in evaluating the biological roles of NO in the central nervous system (172–174). Of significant note is the reported selective inhibition of iNOS by aminoguanidine (175–177). Based on these studies, it is proposed that aminoguanidine may be useful for the treatment of a pathological overproduction of NO without complicating cardiovascular effects.

A recent study reports that the psychomimetic agent, phencyclidine, was capable of irreversibly inhibiting cNOS in a turnover-dependent process (178). It was proposed that phencyclidine can be metabolized by cNOS to a reactive intermediate, which can then covalently bind the protein to irreversibly inactivate it. This finding may have important pharmacological and toxicological implications, since it presents the possibility that NOS can metabolize xenobiotics that may influence not only drug pharmacokinetics but also alter physiological NO levels.

Inhibition of NOS by Arginine Analogs

Analogs of arginine represent the largest and potentially most useful class of NOS inhibitors. Although many of the other, previously mentioned inhibitors

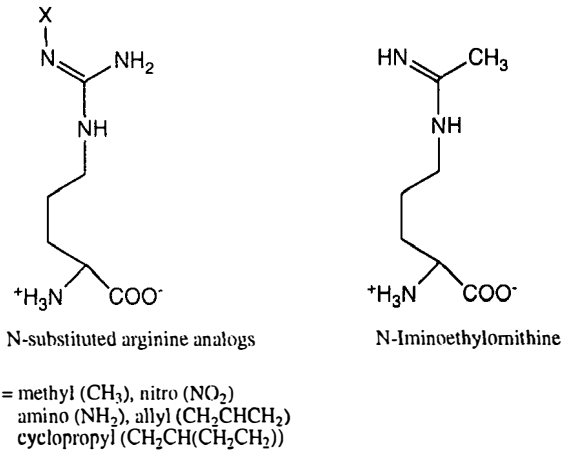


Figure 6 Structures of some arginine analogs used as NOS inhibitors.

of NOS may have similar in vitro potencies compared to arginine analogs, they may be inappropriate as in vivo inhibitors due to the potential for non-specific effects. That is, compounds which act by inhibiting FAD, Ca^{2+}/CaM, heme, H_4B, or thiol function may adversely impact other systems as well, since these cofactors and prosthetic groups are utilized for other purposes throughout the body. Thus, in order to gain a greater degree of functional specificity, it would not be unreasonable to focus on the use of NOS substrate analogs.

A variety of arginine analogs have been found to be inhibitors of NOS activity. The structures of some of these compounds are given in Figure 6. One of the first, most widely utilized and studied of these analogs is N-methyl-L-arginine (33). Early reports indicated that N-methyl-L-arginine was a competitive inhibitor of both iNOS, from macrophages, and cNOS, from endothelial cells (179). However, a closer inspection of the kinetics of N-methyl-L-arginine inhibition of iNOS from macrophages revealed an irreversible component, which was postulated to be mechanism-based enzyme inactivation (180). In that study, one of the postulated mechanisms of inhibition by the N-methyl analog involved iNOS-mediated N-hydroxylation, in a manner similar to that proposed for arginine, to give an N-hydroxy-N-methyl intermediate. This postulate was confirmed in a more detailed study where it was demonstrated that N-methyl-L-arginine undergoes both iNOS (macrophage) and cNOS (rat brain) catalyzed oxidation to generate N-hydroxy-N-methyl-L-arginine, which upon further conversion to citrulline, generates an irreversibly binding species (181). The ability of N-methyl-L-arginine to act as a substrate for NOS (albeit a relatively poor one) was also found in cNOS from porcine

brain (182) and—consistent with earlier work (180, 181)—it was found that N-methyl-L-arginine caused a slow, turnover-dependent enzyme inactivation that correlated with citrulline and NO formation. Thus, it has become apparent that the inhibition of NOS by N-methyl-L-arginine occurs by both competitive and mechanism-based processes.

Another widely used arginine analog whose mechanism of inhibition has been studied is N-nitro-L-arginine. For example, inhibition of endothelium-dependent vasodilation was observed in isolated rabbit aorta and perfused rat mesentary using N-nitro-L-arginine (183). Another study revealed an irreversible component to N-nitro-L-arginine inhibition of brain cNOS both in vivo and in vitro (184). Interestingly, it was also found that the N-nitro analog was a weaker and fully reversible inhibitor of iNOS from macrophages. The selectivity of N-nitro-L-arginine for constitutive NOS (from bovine brain) over inducible NOS (from macrophages) was confirmed in purified systems (185). This detailed kinetic investigation revealed that N-nitro-L-arginine inhibition occurred with a slow onset; and although the association rate was slow (4.4×10^4 $M^{-1}s^{-1}$), an extremely slow dissociation rate ($6.5 \times 10^{-4}s^{-1}$) actually allowed isolation of the cNOS-inhibitor complex. Other studies comparing the mechanism of inhibition by N-nitro-and N-methyl-L-arginine of endothelial cell (186) and brain NOS (182) indicate that both compounds bind NOS in a competitive and reversible manner. However, after incubation times greater than about 3 minutes, the N-nitro analog caused a reversible inactivation of the enzyme. Consistent with the previously described study, it was also found that both the association and the dissociation of N-nitro-L-arginine with NOS was slow (compared to that of the N-methyl analog). Enzyme inactivation by N-nitro-L-arginine was not, however, found to be turnover dependent. Thus, it is apparent that the mechanism(s) of inactivation by N-methyl- and N-nitro-L-arginine are different.

Other N-substituted arginine analogs have also been utilized as inhibitors of both iNOS and cNOS. For example, N-amino-L-arginine is a potent inhibitor of endothelium-dependent relaxation (187) and endothelial cell NO production (179), and raises vascular resistance during septic shock (188). N-iminoethyl-L-ornithine has been used as an irreversible inhibitor of NOS from phagocytic cells (189) and in the vascular endothelium (190). N-allyl and N-cyclopropyl-L-arginine were synthesized as possible mechanism-based enzyme inhibitors (191). Although the N-cyclopropyl analog was found to be only a reversible inhibitor of iNOS from macrophage, the N-allyl derivative exhibited both reversible and irreversible behavior.

Several studies have focused on the cell and tissue selectivity of arginine analogs, for example, selective inhibition of iNOS in macrophages and cNOS from endothelial cells by N-amino-, N-methyl-, and N-nitro-L-arginine (167). It was found that the N-amino and N-nitro analogs were about 100-fold more

potent than the N-methyl analog in blocking NO synthesis in endothelial cells. On the other hand, the N-amino and N-methyl analogs were equipotent in inhibiting macrophage iNOS, whereas the N-nitro analog was much less potent. Thus, N-nitro-L-arginine appears to exhibit significant selectivity for inhibition of cNOS. In another study, the inhibitory potencies of N-nitro-, N-methyl-, and N-amino-L-arginine were examined in three cell systems (192). In iNOS from macrophages, the rank order potency for inhibition of NO biosynthesis was found to be N-amino ≥ N-methyl >> N-nitro. However, for inhibition of cNOS in CNS tissue or endothelial cells, the rank order potency was N-nitro >> N-amino ≥ N-methyl. Again, the N-nitro analog shows selectivity for the cNOS isoforms over iNOS. Inhibition of cNOS in the CNS and iNOS in macrophages and smooth muscle by two novel arginine analogs—N-amino-L-homoarginine and N-cyclopropyl-L-arginine—were compared (193). The N-cyclopropyl analog exhibited a 300--500-fold selectivity for cNOS, whereas N-amino-L-homoarginine blocked NO production from all the cells with similar potency. Taken together, these studies indicate that some isoform selectivity can be accomplished by simple derivatization of L-arginine.

SUMMARY: POTENTIAL FOR NOS ISOFORM-SELECTIVE INHIBITION

Although the inhibition of either iNOS or cNOS can be accomplishd utilizing a number of agents (some of which have been mentioned herein), it remains to be seen if any of these strategies will be of any therapeutic value. The diverse and somewhat unrelated physiological functions of NO make it a difficult therapeutic target. That is, alteration of NO levels for one purpose may adversely affect another, unrelated function. Therefore, it would be advantageous to be able to selectively inhibit NO generation in one tissue or cell type without significantly altering the levels in the others. Fortunately, NO biosynthesis does not occur through the actions of a single NOS enzyme but is the result of a variety of different isoforms which differ in both tissue and cellular location, depending on their intended function. Thus, isoform-selective inhibition of NOS may result in the selective lowering of NO in the desired tissue or cells. For example, endotoxin or septic shock is the result of an overstimulation of iNOS, so elective inhibition of iNOS would be a possible strategy in the treatment of this problem. The regulation of iNOS and cNOS are fundamentally different and may offer a way for selective manipulation of isoform activity. However, it should be realized that manipulation of NOS regulatory factors—such as Ca^{2+} or CaM—may impact other, unrelated physiological events that also utilize these factors. Therefore, it would seem most reasonable that selective in vivo NOS isoform inhibition will be accomplished utilizing substrate analogs. That is, by taking advantage of the specificity of NOS for

L-arginine, it may be possible to develop inhibitors which are isoform selective. This, of course, presumes that the iNOS- and cNOS-binding parameters for L-arginine differ. This appears to be the case, based on several of the above-mentioned studies (167, 192, 193). That is, selective inhibition of the cNOS appears to be possible utilizing, for example, N-nitro-L-arginine. Although selective inhibition of iNOS over cNOS is not usually observed with the arginine analogs, N-aminoguanidine has been found to be a fairly selective inhibitor for the iNOS isoform. Since aminoguanidine appears to be competing with L-arginine for the active site (176), this result further supports the likelihood that the arginine-binding domains for the iNOS and cNOS isoforms are different and may be exploited for therapeutic purposes. It should be mentioned that the iNOS and cNOS designations used herein are a bit oversimplistic. There are a variety of cNOS and iNOS isoforms that may all have different or distinct binding characteristics for both substrate and cofactors. However, more work will have to be done to determine whether these differences will be substantial enough to allow for their selective inhibition or manipulation.

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