JOURNAL OF
MEDICINAL
CHEMISTRY

Volume 37, Number 13 June 24, 1994

Perspective

Approaches toward Selective Inhibition of Nitric Oxide Synthase

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Received October 7, 1993

Michael A. Marletta received his A.B. degree in chemistry from the State University of New York, College at Fredonia, and his Ph.D. in Pharmaceutical Chemistry from the University of California, San Francisco, where he used both chemical and biochemical approaches to the study of the mechanism of creatine kinase. From 1977 to 1987, Dr. Marletta was at MIT, firstly as a postdoctoral fellow in Christopher Walsh's laboratory and subsequently as a faculty member in the Department of Applied Biological Sciences. In 1987 he joined the College of Pharmacy at the University of Michigan, Ann Arbor, where he is presently Professor of Medicinal Chemistry in Pharmacy and Biological Chemistry in the Medical School and John G. Searle Professor of Medicinal Chemistry. Dr. Marletta's primary research interests lie at the interface of biology and chemistry with especial emphasis on enzymatic reaction mechanisms, particularly those of nitric oxide synthase and guanylate cyclase.

The intense interest and rapid pace of nitric oxide (•NO) research has occurred, at least in part, because of the significant therapeutic potential associated with the rational control of •NO synthesis. Several recent reviews have appeared that highlight various aspects of this area of research.[1-4] The biological actions of •NO are directly related to the tissue and cell type that produces it. For example, when •NO is synthesized in the vascular endothelium, it causes relaxation of the adjacent smooth muscle via activation of guanylate cyclase, an enzyme present in vascular smooth muscle. The resulting transient increase in cGMP acts as a second messenger in this relaxation, presumably via a cGMP-dependent kinase. A similar mode of action has been proposed for cell to cell signaling in the brain, and although there is not uniform agreement on the function of •NO in the brain, it appears to play some role in long-term potentiation.[5] As mentioned below, some if not most nonadrenergic, noncholinergic (NANC) neurons are •NO-activated nerves, and the signaling in these neurons has also been speculated to involve cGMP. •NO has also been shown to be synthesized by immune system cells such as macrophages and neutrophils where a function in the host response to infection has received considerable attention. The immune system seems to have harnessed the toxic properties of •NO to kill or stem the growth of invading organisms that have been reported to include various pathogens and tumor cells.[6] The exact mechanism of killing or growth stasis is a difficult question, but increasing evidence supports the hypothesis that •NO interferes with iron homeostasis and key cellular iron proteins.[7] Since the initial report of •NO-induced ADP ribosylation,[8] several additional papers have appeared reporting that •NO caused an auto ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[9-11] However, it appears that the reaction does not involve a straightforward ADP ribosylation of GAPDH. A reaction involving •NO, the nicotinamide ring of NAD, and the active-site thiol of GAPDH has been reported to occur.[12] The therapeutic potential of •NO has apparent application in situations where levels of •NO are elevated above what are considered normal for physiological function and in situations where the •NO concentration is presumably below that which leads to the desired response. Since a number of reviews have been written on both the established and potential physiological actions of •NO, this article will focus on approaches toward regulation of •NO action by control of nitric oxide synthase (NOS), the enzyme responsible for •NO synthesis. While strategies to increase NOS activity *in vivo* are possible, the use of •NO-releasing drugs represents a more realistic approach toward resolving clinical problems that are related to low levels of •NO or problems that can be reversed by direct treatment with •NO.

Inhibition of NOS, in particular isoenzyme selective

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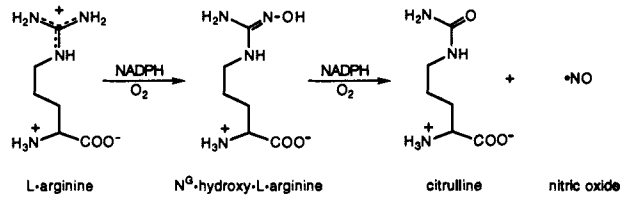

Figure 1. Reaction catalyzed by nitric oxide synthase. Also shown is N^G-hydroxy-L-arginine (L-NHA) which can substitute for L-arginine and appears to be an intermediate in the reaction.

inhibition, will clearly be of therapeutic value. This is especially evident in endotoxic shock where levels of •NO exceeds normal physiological levels. The clinically observed extreme hypotension in shock has been linked to elevated levels of •NO and has been reversed in both animal models of shock[13] and in humans.[14] Early results show, however, that selective inhibition will be critical in the treatment of shock.[15] Inflammation associated tissue damage has also been linked to elevated levels of •NO[16] and may be related to the formation of peroxy nitrite and the reactivity of this oxidant.[17] It has also been speculated that postischemic damage in stroke may be related to increased •NO levels that presumably would occur. The origin of this increase is speculated to result from the rise in Ca^{2+} and the resulting stimulation of NMDA receptors which in turn would activate •NO synthesis.[18]

On the other hand, evidence is accumulating that relates certain clinical problems to the lack of, or diminished levels of, •NO. Although the lack of •NO or NOS has not be explicitly shown, treatment with •NO or •NO-releasing drugs either is being used or is under consideration. In particular, nonadrenergic, noncholinergic (NANC) neurons appear to be regulated by •NO, apparently providing a role for •NO in gut motility and penile erection for example. Indeed studies have shown that •NO plays a critical role in erectile function in the penis.[19,20] Perhaps most surprising is the relatively rapid move that this area of research has made into the clinic where infants and adults with pulmonary hypertension have been successfully treated with •NO added to ventilator gas.[21,22] Several other diseases have been implicated and include rheumatoid arthritis[23] and inflammatory bowel disease.[24] As investigations continue, novel physiological functions of •NO will be uncovered. For example, the recent reports that inhibition of •NO synthesis prevents tolerance to morphine is a particularly interesting and potentially important new development.[25,26] Evidence for a role in gene transcription has also been recently reported.[27] Although direct treatment with •NO is beyond the scope of this review, it is worth noting that the toxicity and clinical reactivity of •NO may limit its applicability.

Characterization of Nitric Oxide Synthase

Considerable progress has been made in the characterization of NOS.[1] The reaction catalyzed by this enzyme is shown in Figure 1 and involves the oxidation of L-arginine to citrulline and •NO. The reaction is overall a five-electron oxidation. A number of NOS isoforms have been purified and characterized, and several more have been cloned and, in some cases, functionally expressed. The picture that is emerging is one of a family of closely related proteins, at least some of which are the products of several distinct genes. In general, the synthases can still be grouped into two broad categories: (i) a constitutive, Ca^{2+}/calmodulin-dependent type that is involved in cellular signaling and (ii) an inducible isoform characterized in macrophages and other cells that sometimes shows a dependence on Ca^{2+}/calmodulin. The first constitutive NOS isoforms purified were from rat and porcine cerebellum.[28-30] These proteins, with reported monomeric M_r values on SDS–PAGE ranging from 150 to 160 kDa, are cytosolic and exist as homodimers under native conditions. A constitutive isoform (135 kDa on SDS–PAGE) isolated from bovine vascular endothelium is distinct in that it is membrane bound.[31] The cDNA-derived amino acid sequence of this endothelial cell NOS isoform shows a myristoylation consensus sequence at the N-terminus which is absent in the cytosolic isoforms isolated from murine macrophages and rat cerebellum.[32] In addition, the bovine endothelial NOS sequence showed no transmembrane domain regions, suggesting that myristoylation is responsible for the membrane anchor of this NOS isoform. An initial report was consistent with myristoylation of the endothelial NOS,[33] and subsequent studies convincingly showed that myristoylation is critical for the membrane association of this isoform.[34]

The inducible isoform purified from murine macrophages is a cytosolic protein with a monomeric M_r of 130 kDa and again found to be dimeric under native conditions.[35,36] A sequence comparison shows that this isoform is about 50–60% identical to the cerebellar isoforms. In general, isoforms from the same tissue are essentially identical. For example, the sequence comparison of the bovine endothelial to human endothelial shows them to be 95% identical,[32,37] whereas both of these isoforms show 50–60% identity to the macrophage inducible isoform. The rat hepatocyte inducible isoform is essentially identical (96%) to the murine macrophage isoform.[38] An interesting picture is emerging with regard to the comparison of inducible isoforms. Several human inducible isoforms have been cloned including one from hepatocytes[39] and one from a colorectal adenocarcinoma cell line.[40] These isoforms are essentially identical to each other (~99%) with only a few amino acid differences. However, comparison of these inducible isoforms to that from the murine macrophage shows about 80% identity. In general, while all human isoforms are essentially identical regardless of the origin of the tissue, significant differences exist with the murine macrophage isoform. Nonetheless, polyclonal antibody raised against the murine macrophage NOS cross-reacts with human liver inducible NOS, but not with constitutive isoforms (Richards and Marletta, unpublished results). A truncated mRNA coding for the rat cerebellar NOS has been isolated recently.[41] The significance of this finding is not clear, especially since the region missing comes about from an in-frame deletion of two exons. These exons are from a highly conserved region of the all the NOSs and may represent the substrate binding site.

The cofactor binding sequences are highly conserved in all the isoforms. Bredt et al. were the first to report isolation of a cDNA for the rat cerebellar NOS and the first to note the significant homology to NADPH cytochrome P-450 reductase from the derived amino acid sequence.[42] This P-450 reductase domain appears to be present in all isoforms and has the requisite binding sequences for NADPH, FAD, and FMN. As the sequence predicts, 1 equiv each of FAD and FMN were found bound to the murine macrophage NOS.[35] In general, this has been found to be true for other isoforms that have been examined, although several reports note a requirement

for FAD or FMN which is most likely due to loss of the flavin during purification. Additionally, NOS is now known to contain a cytochrome P-450 type iron protoporphyrin IX heme. This was initially found for the macrophage isoform[43] and subsequently in other isoforms.[44,45] The λ_{max} of the Soret peak is ~400 nm and that of the reduced CO complex is ~445 nm. The heme domain of the protein is probably located toward the N-terminus since from the sequence homology the reductase domain is clearly located in the C-terminus. However, the N-terminal sequence shows little homology to the P-450s, and it appears that NOS lies outside the large group of related P-450 gene products referred to as the P-450 superfamily.[46] The common P-450 structural elements are missing in NOS, suggesting that NOS is more likely to be an example of convergent evolution rather than a protein that diverged from the common P-450s.[46] Typical P-450 substrates are very lipophilic compounds with residues in the active site designed to accommodate these type of substrates. Although nothing is known at this point about the active site of NOS, given the polar nature of the substrate L-arginine, it is likely to be dramatically different when compared to typical P-450s. Hence the lack of sequence similarity at the N-terminus when NOS and P-450s are compared is not too surprising.

In addition, the common sequence that is found in most P-450s and involves the cysteine residue that ligates to the heme-iron also appears to be absent, so while spectrally characterized as a P-450, NOS clearly is somewhat atypical for this highly conserved class of proteins. Nevertheless, some of the chemistry catalyzed by the enzyme that is described below is typical of P-450s. Taken together, these findings, plus the fact that NOS contains a P-450 reductase domain, place NOS is a unique biochemical niche as a so-called self-sufficient P-450. Only one other example exists of a P-450 that has the reductase in the same polypeptide as the heme, and that is the fatty acid monooxygenase from *Bacillus megaterium* characterized by Fulco and co-workers.[47] These investigators in fact coined the term self-sufficient P-450.

NOS also shows a dependence on (6*R*)-tetrahydro-L-biopterin (H_4B) which is typically used as a cosubstrate in the aromatic amino acid hydroxylases such as phenylalanine hydroxylase where it serves to provide the two electrons needed for oxygen reduction. While a specific role for H_4B in the NOS reaction is not clear at this time, several important findings have been reported. Unfortunately, the gross details of the NOS-catalyzed reaction will remain incomplete until the function of this reduced pterin is understood. The H_4B requirement for NOS was first observed in studies with the inducible murine macrophage isoform[48,49] and has subsequently been shown for all NOS isoforms.[31,50-52] The picture that is emerging suggests an unusual role for this reduced pterin in the NOS reaction. Although what follows has been carried out with inducible isoform, the results will probably be similar with the constitutive isoforms. H_4B forms a 1:1 complex with NOS.[53] The reduced pterin will dissociate from the enzyme especially if the purification is carried out without H_4B in the buffers.[53] The amount of pterin that dissociates must be empirically determined, but NOS isolated in this manner is typically about 20–50% saturated with pterin and the activity is linearly dependent on the pterin bound.[53] To date several interesting effects of H_4B have been observed. Macrophage NOS isolated in the absence of H_4B has a significantly altered Soret peak of the heme spectrum. The λ_{max} of this so-called pterin-deficient NOS is ~418 nm, indicative of a low-spin-state heme.[54] As mentioned above the pterin-saturated NOS λ_{max} is ~400 nm. NOS assayed in the presence of H_4B is more tightly coupled as well.[55,56] These results suggested that H_4B had an effect on the structure of the enzyme and led to the examination of the influence of H_4B on the monomer (130 kDa) to dimer (260 kDa) ratio, since all NOS isoforms appear to be homodimers in the native state. Indeed, using native gels to analyze the monomer:dimer ratio, a significant portion of pterin-deficient NOS exists in the inactive monomeric state and that the ratio is shifted to dimer with the addition of H_4B.[54] Although this dimerization is dependent largely upon H_4B, a recent report that examined essentially the same question concluded that dimerization requires heme, L-arginine, and H_4B.[57] This latter report shows that activity is primarily associated with the dimeric form of the enzyme.

Current thoughts on the mechanism have recently been reviewed.[1,58] Briefly, the first step in the reaction involves the hydroxylation of L-arginine to produce N^G-hydroxy-L-arginine (L-NHA).[59,60] The molecular details involved in the further conversion of L-NHA are not known; however, recent results are consistent with heme involvement in this step as well as the step that leads to the hydroxylation of L-arginine.[61] The formation of L-NHA is thought to entail the straightforward P-450-catalyzed hydroxylation of L-arginine. While P-450-dependent hydroxylation of guanidines has yet to be established, precedent for this type of hydroxylation on amidines has been reported.[62] The conversion of L-NHA to citrulline and •NO has been speculated to involve a ferric peroxide heme intermediate as a nucleophile in the reaction in a manner analogous to that proposed for aromatase. The involvement of a ferric peroxide nucleophile in P-450 reactions was first proposed by Akhtar and colleagues in estrogen biosynthesis.[63,64] The demethylation of the C-19 position involves 3 equiv of NADPH and O_2. The first two steps are considered to be straightforward hydroxylations ultimately forming the C-19 aldehyde. The third step is proposed to involve the ferric peroxide intermediate. This is particularly interesting since this would necessitate an apparent change in mechanism where chemistry takes place before oxygen–oxygen bond scission occurs. Furthermore, if L-NHA provides one of the two electrons needed, then the attack of the heme-ferric peroxide on this amino acid radical intermediate should lead directly to the products of the reaction.[1] Recently reported chemical model studies support this type of mechanism in NOS.[65] Up to this point the structural differences noted above for the various NOS isoforms has not translated into significant differences in terms of mechanism. The homology identity that has been observed in all isoforms in the important regions of the protein does not auger well for the development of selective inhibitors that are based on substrate structure. And yet, as described below, surprisingly this potential does indeed exist.

Drug Design

A number of strategies have emerged with regard to the rational control of the physiological levels of •NO. By far, inhibition of NOS has received the most attention, and as mentioned above isoform-selective inhibitors will be essential in any therapeutic application. In particular, most efforts have been directed toward selective inhibition

of the inducible NOS that could be used in the treatment of endotoxic shock and other clinical problems related to abnormally high levels of •NO. As described above, several important differences exist among the isoforms suggesting, at least in principle, that isoform-selective inhibition is possible. Strategies include (i) interaction at the arginine binding site, (ii) ligands directed toward the heme, (iii) interaction at the H_4B binding site, (iv) inhibition of H_4B biosynthesis, (v) Ca^{2+}/calmodulin antagonism, and (vi) interference in the transcriptional regulation mechanism of inducible NOS.

(i) Arginine Analogues. All of the NOS isoforms have shown rather narrow specificity at the arginine binding site. The requirement for the L-configuration at the α-carbon appears to be absolute exemplified by that fact that D-arginine is not a substrate and does not inhibit the reaction. This same stereochemical rigidity has been observed for several arginine analogues that have been studied as potential substrates or inhibitors. Other than L-arginine, only L-homoarginine has been reported to be a substrate for the reaction.[66] The homologs of L-arginine that are shorter by successive carbons have not been studied in detail. As discussed below, N^G-methyl-L-arginine, the widely studied NOS inhibitor, can also serve as an alternate substrate.[67]

Hibbs and colleagues were the first to describe the use of N^G-methyl-L-arginine (L-NMA), a simple *N*-alkyl-substituted arginine derivative, to inhibit •NO formation.[68] These studies in macrophages were initially focused on the ability of these activated cells to kill invading organisms such as tumor cells and pathogens. While these cytotoxicity oriented investigations were being carried out, parallel experiments probing a role of macrophages in nitrosamine-induced carcinogenesis were on-going, and both lines of investigations led to the characterization of a novel macrophage metabolic pathway involving the conversion of L-arginine to citrulline, NO_2^-, and NO_3^-.[68-71] LPS and cytokines such as IFN-γ induce the synthesis of NO_2^- and NO_3^- in murine peritoneal macrophages in culture as well as from a variety of established macrophage cell lines.[69,70,72] It was in this context that L-NMA was used to inhibit NO_2^- and NO_3^- synthesis, prior to the finding that •NO was an intermediate in the reaction which then generated NO_2^- and NO_3^- as the stable solution decomposition products of the reaction. Since this initial finding and the subsequent results which showed that L-NMA directly inhibited •NO formation in murine macrophages,[73] many reports have appeared that have used this and other simple *N*-alkyl derivatives as NOS inhibitors.

Most of the reported studies have been carried out with crude, partially purified NOS or cells in culture and consequently must be interpreted with caution. In general, all simple arginine analogues inhibit both the inducible and constitutive NOS isoforms. These compounds are presumed to be reversible competitive-type inhibitors since most of the *in vivo* studies which involve measuring a physiological effect in response to treatment with an arginine analogue, followed by washout of the inhibitor with a buffer that contains arginine, observed the subsequent return of the inhibited response. These results suggest that, at least with the constitutive form of the NOS, the inhibition is reversible. However, as discussed below, this is not always the case.

Some attempts have been made to rank-order the potency of various analogues. Gross and colleagues

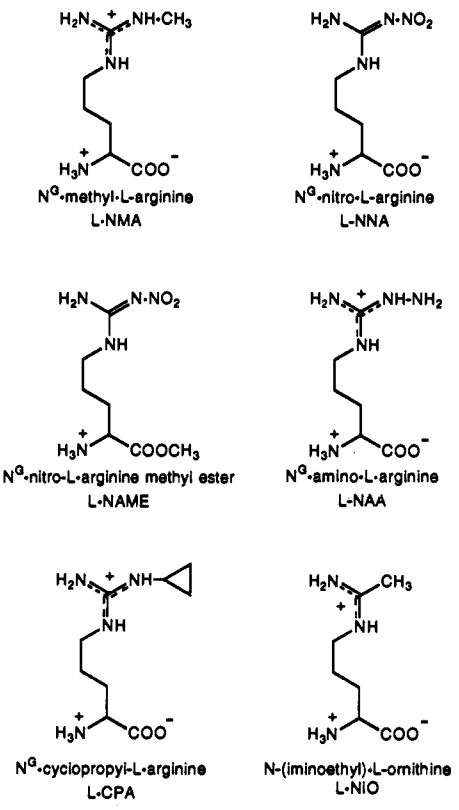

Figure 2. L-Arginine analogues characterized as NOS inhibitors.

examined L-NMA as well as N^G-amino- and N^G-nitro-L-arginine (L-NAA and L-NNA, respectively).[74] The studies were carried out in cell culture with activated murine macrophages and bovine aortic endothelial cells and measured only NO_2^- in the media. They found N^G-amino- and N^G-nitro-L-arginine to be about 100-fold more potent than L-NMA in inhibiting endothelial NO_2^- synthesis with IC_{50} values of 1 and 100 μM, respectively. On the other hand, L-NMA and N^G-amino- were about equipotent in inhibiting macrophage NO_2^- synthesis and N^G-nitro- much less active (IC_{50} values of 4.3, 2.6, and 60 μM, respectively). Differences due to uptake by the cells were not addressed, and this would be expected to play an important role in the overall potency. This is especially true because L-NMA appears to be transported by the cationic amino acid transporter while *N*-alkyl analogues with substitutions that greatly lower the pK_a of guanidine moiety, such as N^G-nitro, are transported as neutral amino acids.[75] Similar comparisons have been generated by others, and while there have been qualitative differences, in general N^G-nitro-L-arginine appears to be relatively more potent for constitutive isoforms of NOS.[76,77] Many *in vivo* studies have employed the methyl ester of N^G-nitro-L-arginine (L-NAME). In some cases this analogue appears to be biologically more potent, although the reason for this is not clear. It is possible that the ester enables this analogue to cross the cell membrane more easily; however, once inside ester hydrolysis almost certainly occurs at a significant rate. In short, it is not clear if the ester itself is active or simply liberates the carboxylic acid inside the cell.

Several other alkyl analogues have been synthesized and studied to a limited extent. For example, N^G-cyclopropyl-L-arginine (L-CPA) was found to be a reversible inhibitor of the inducible NOS from murine macrophages.[78] This analogue showed modest selectivity for the constitutive isoform in studies carried out in cellular

systems that compared cytokine-induced •NO synthesis in murine macrophages, vascular smooth muscle versus constitutive •NO synthesis in central nervous system tissue yielding IC_{50} values of 184, 258, and 0.55 μM, respectively.[77] Whether this represents true selectivity at the active site or some other phenomenon remains to be established. N^5-(Ethylimino)-L-ornithine (also known as *N*-(iminoethyl)-L-ornithine) (L-NIO) has also been examined in cellular systems and homogenates and compared to L-NMA and L-NNA.[79] While L-NIO was about 5 times more potent than L-NMA and L-NAME in indirect measures of NOS activity, in physiological type experiments L-NAME was the most potent in inhibiting acetylcholine-induced relaxation of rat aortic rings and more potent in lowering of mean arterial blood pressure and heart rate.[79] Although quite interesting, these results cannot yet be interpreted on a molecular level.

All the findings summarized above have characterized reversible inhibition of NOS. Several reports have appeared that have described irreversible inhibition of NOS. This irreversible inhibition has been most thoroughly characterized with the inducible murine macrophage NOS and L-NMA.[67,80,81] The initial report of irreversible inactivation was carried out with unpurified murine macrophage NOS.[80] L-NMA showed time and concentration dependent inactivation of NOS that was protected by L-arginine. Furthermore, this inactivation appeared to be dependent on turnover, suggesting that L-NMA is acting as a mechanism-based inhibitor of NOS. It had been proposed that turnover involved N-hydroxylation at the substituted nitrogen generating N^G-hydroxy-N^G-methyl-L-arginine (L-NHMA) as a product.[80] This compound was synthesized and found itself to inactivate the purified macrophage NOS in a mechanism-based fashion.[60] From a comparison of the macroscopic kinetics derived from the inactivation experiments, L-NHMA is more effective than L-NMA.[60,67] Similar results with L-NMA and L-NHMA were obtained by Feldman and colleagues in a comparison of irreversible inactivation of murine macrophage and rat cerebellar NOS[81] and by Mayer and co-workers.[82] The turnover of L-NMA with purified NOS has recently been characterized more thoroughly[67,81] and is illustrated in Figure 3. Briefly, the initial step is most easily understood in terms of typical P-450 oxidative chemistry where after initial $1e^-$ oxidation, oxygen rebound can take place on either N or C. When oxygen rebound occurs on the methyl C, L-NMA is demethylated forming CH_2O and L-arginine.[67] NOS that has not been inactivated will then convert the *in situ* generated L-arginine to •NO and citrulline. As initially proposed, L-NHMA is also observed as a reaction product,[60,67,81] presumably via oxygen rebound on N. NOS will convert L-NHMA to •NO and citrulline as well, along with the formation of CH_2O, which has led to the speculation of a nitrone intermediate[67] (Figure 3). If formed, this nitrone should hydrolyze to the observed products, and it is a likely intermediate considering the potential chemistry that could occur at the active site. It is clear that this processing is directly related to the inactivation observed; however, exactly how is still an open question. The formation of a methylnitrosonium ion has been proposed,[81] although it is unlikely that this species, if formed, will be solely responsible for the inactivation. It is interesting that NOS apparently acts only on the substituted nitrogen atom. Formation of products derived from the unsubstituted nitrogen have not been observed.

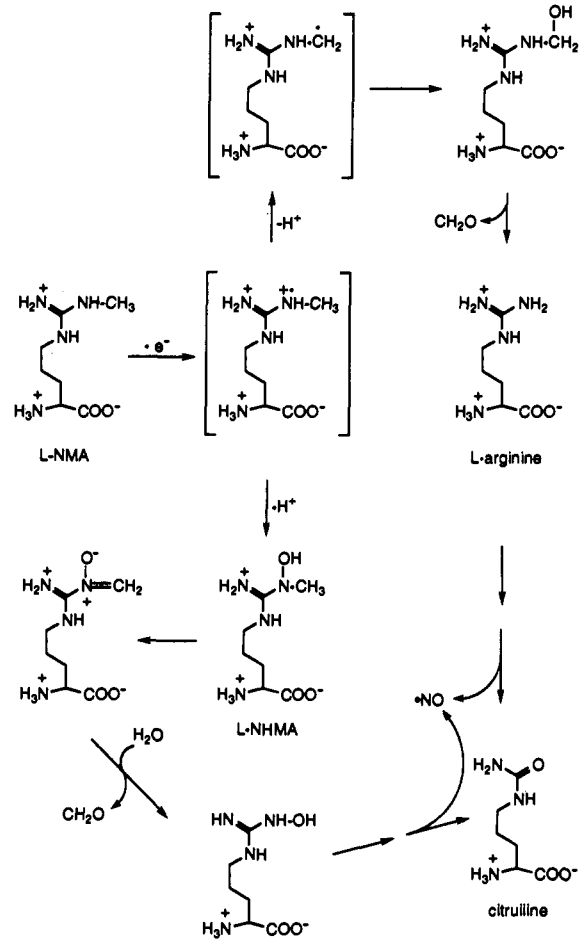

Figure 3. N^G-Methyl-L-arginine (L-NMA) processing by NOS. The reactions characterized are consistent with NOS functioning as a cytochrome P-450.

Several other studies have reported observations that suggest mechanism-based inactivation of NOS. The apparent irreversible loss of cerebellar NOS activity in rats treated with N^G-nitro-L-arginine (L-NNA) has been reported.[83] At about the same time, Moncada and colleagues described the inhibition of NOS activity in cells and supernatant derived from either the murine macrophage J774 cell line or rat peritoneal neutrophils by *N*-(iminoethyl)-L-ornithine (L-NIO).[84] While L-arginine could protect against this inhibition if co-incubated with L-NIO, the inhibition could not be reversed by L-arginine, suggesting that irreversible inhibition had taken place. A recent report compared the inhibition observed with L-NNA with the constitutive rat cerebellar NOS and the inducible murine macrophage NOS. This analogue was found to be a simple reversible inhibitor of the inducible NOS; however, it was about 300-fold more potent in inhibiting the constitutive NOS.[85] More interesting was the finding that the inhibition of the constitutive NOS appeared to be of a slow onset, tight-binding type of inhibition. Similar results with L-NNA and the rat cerebellar NOS have been reported by others as well.[82] Both reports, however, do not address their findings with respect to the stoichiometry of L-NNA bound versus the level of inactivation.[82,85] They do not appear to be directly correlated as expected, implying that some additional interaction or chemistry is occurring. These results provide an explanation for the observations with L-NNA

in rats and shed further light on active-site differences that exist between these two isoforms. The potential for the design of arginine-based analogues that show selective NOS isoform inhibition clearly exists.

(ii) Heme Ligands. NOS contains a cytochrome P-450 type heme,[1] and one of the general approaches that has been used for this class of enzymes are compounds that could form ligands to the heme. Destruction of the heme after binding is also an approach toward irreversible inhibition, and this may play a role in the inhibition of NOS by N^G-amino-L-arginine. The inhibitory properties of aminoguanidine may also rely on the ability to coordinate with heme,[86] since guanidine itself does not inhibit the enzyme. Imidazole derivatives such as the antimycotic agents miconazole and ketoconazole inhibit P-450s and show a binding spectrum consistent with interaction at the heme binding site. Recently, Wolff and colleagues showed that the cerebellar NOS is inhibited by imidazole, 1-phenylimidazole, and 2-phenylimidazole as well as miconazole, ketoconazole, and clotrimazole.[87,88] The inhibition (IC_{50}) with the imidazole and the phenyl-substituted imidazoles ranged from 25 to 600 μM, while the antimycotic agents were more potent ranging from 0.8 to 20 μM. In addition to potential interaction at the heme site, the antimycotic imidazoles seemed to interfere with the calmodulin–NOS interaction. Imidazole itself does form a ligand to the heme in the macrophage NOS (White and Marletta, unpublished results) and rat cerebellar NOS;[89] therefore, the mode of action of these compounds as heme ligands is a potential mechanism of inhibitory action. Initial studies with 7-nitroindazoles as inhibitors may also be explained based on this type of interaction.[90] On the basis of the results summarized above, inhibitor design based on heme ligation is likely to meet with some success, albeit the path toward the design of selective inhibitors based on this strategy is presently not clear.

Recently, some attention has been focused on the possibility that the •NO derived from NOS inhibits subsequent turnover of the enzyme in a manner analogous to feedback inhibition.[91] The ability of •NO to form a relatively stable complex with both ferric and ferrous P-450 hemes has been established a number of years ago;[92] however, the complexes were unstable in the presence of oxygen. In general, the ferric complexes of hemoproteins and •NO have fast off rates, and so the instability is due to dissociation followed by reaction of •NO with O_2 in solution. The ferrous nitrosyl complexes typically have slow off rates; however, most of these studies have been carried out with hemoproteins such as hemoglobin where the ligand to the heme is histidine and not cysteine as in P-450 and NOS. Issues related to this topic have recently been discussed.[93] While the formation of a nitrosyl complex has been observed during turnover (Hurshman and Marletta, unpublished observations), it forms only under conditions of relatively high enzyme concentration and is spectrally transient. The potential of •NO to inhibit NOS physiologically is still an open question; however, the chemistry of •NO interaction with a P-450 type heme[92] and the reactivity of •NO with ferrous oxy heme complexes such as hemoglobin[94] argues against this potential mode of inhibition. The analogy to CO inhibition is probably not relevant since the interactions of CO with either type of heme is fundamentally different and the CO complexes with both types of heme are chemically stable under oxygen.

(iii) H_4B Binding Site/Biosynthesis. As briefly described above, the role H_4B in the NOS reaction is not entirely clear, despite the overall importance of this reduced cofactor to the enzyme. Interference with either H_4B binding or H_4B biosynthesis is potentially a target for drug design because of the inherent instability of NOS in the absence of H_4B.[53,54] Inhibition via the reduced pterin function/binding site will most likely be of some use with inducible NOS. Most attention has been directed toward inducible NOS in the vascular smooth muscle where elevated levels of •NO are presumed to play a role in the hypotension associated toxic shock. Indeed, a dependence on H_4B has been shown for induction in the vascular smooth muscle,[95] and this appears to be related to *de novo* synthesis.[96] Several years ago, a substantial effort was invested into H_4B replacement therapy in an effort to treat several diseases such as atypical phenylketonuria, Parkinson's disease, and a number of neurological disorders that were throught to involve low *in vivo* levels of H_4B. The results of these studies have been mixed. If H_4B-targeted approaches toward NOS are going to be effective, selectively will be the critical issue. The previous studies with diseases that involve abnormal H_4B levels illustrate the potential problems of nonspecific effects. Since the interaction of NOS with H_4B is quite different from that when it functions as a recyclable in solution cosubstrate, it is possible that an understanding of the binding site will point the way toward a selective acting pterin antagonist. Efforts to identify the pterin binding site are underway and will provide details into H_4B binding and, therefore, the design of agents to interact at this site.

(iv) Ca^{2+}/Calmodulin Antagonists. Inhibition of NOS through calmodulin antagonists should, in principle, be fraught with selectivity problems and provide an unfruitful avenue in the design of NOS inhibitors. This will almost certainly be the case for constitutive NOS isoforms that show a typical type of calmodulin interaction and have been shown to be inhibited by Ca^{2+} chelators such as EGTA and calmodulin antagonist such chlorpromazine and W-7. Namely, a reversible binding to NOS dictated by increases in intracellular Ca^{2+} levels. Inducible NOS isoforms from the outset showed no dependence on either Ca^{2+} or calmodulin. This view is beginning to change, brought about initially from analysis of the cDNA sequence.[97-99] All three groups noted that a calmodulin recognition sequence appeared to be present in the murine macrophage NOS. Indeed, it appears that calmodulin copurifies with the inducible NOS from murine macrophages.[100] This study suggests that inducible NOS isoforms form a very tight (essentially irreversible) interaction with calmodulin and does so requiring very low concentrations of Ca^{2+}. As with H_4B described above, since the interaction of calmodulin with NOS appears to be unique, the design of agents that will selectively interfere with this process is possible. Interestingly, the characterization of other inducible NOS isoforms have shown that some show an interaction with calmodulin that is more typical. For example, human hepatocyte inducible NOS (cloned and functionally expressed)[39] and rat hepatocyte inducible NOS (purified)[101] both show inhibition by EGTA and calmodulin antagonists. Comparison of the presumed calmodulin binding sequence in these cloned forms shows some nontrivial substitutions compared to the macrophage sequence. Significant progress has been made recently on calmodulin–NOS interactions, with the rat cerebellar

isoform using peptides derived from the calmodulin binding site.[102,103]

(v) Transcriptional Regulation of Inducible NOS. The regulation of inducible NOS is currently under study, and it is not clear at this time if a viable approach toward inhibitor design will emerge. Initial reports of NOS genomic sequence analysis indicates that control of enzyme expression is complex. Interestingly, simple glucocorticoids may function by inhibition of expression of inducible NOS in vascular smooth muscle.[104] Since glucocorticoids do not directly inhibit the enzyme, it does appear that the inhibition was the result of inhibition of expression. Other avenues to approach inhibition at the level of transcription or translation await further understanding of the mechanisms of regulation. Complete pharmacological inhibition of a specific NOS isoform is, at present, not possible. However, specific disruption of the neuronal NOS gene, commonly referred to as a "knock-out" experiment, was recently reported in mice.[105] Expression of this NOS isoform and NADPH-diaphorase staining that is correlated with NOS activity were both absent in the mutant mice. Interestingly, neuronal morphology, viability, and other characteristics examined appeared normal, although low levels of NOS activity in the brain were observed. The mice did develop greatly enlarged stomachs as well as other gastrointestinal abnormalities. This type of approach will no doubt prove quite useful in dissecting the complex signaling pathway mediated by •NO.

Conclusions

There is no question that •NO occupies an important physiological niche and that imbalances in the finely tuned synthesis can have extremely deleterious ramifications. The rapid advances made in the understanding of NOS enzymology has already suggested strategies that can be employed toward the design of potent inhibitors that could be used in clinical situations related to elevated levels of •NO. The debate continues over disease targets beyond endotoxic shock, but it is likely that others will emerge and that controlling •NO at the molecular levels will involve the design of isoform-selective NOS inhibitors.

Acknowledgment. The author is extremely grateful to the students and post-doctoral fellows that have made invaluable contributions to the studies reported that originated in the author's laboratory. Support from the NIH (CA 50414, CA 26731), The Hitchings Award of the Burroughs Wellcome Fund, and Parke Davis Pharmaceutical Research Division is gratefully acknowledged.

References

(1) Marletta, M. A. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **1993**, *268*, 12231–12234.
(2) Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **1992**, *6*, 3051–3064.
(3) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109–142.
(4) Kerwin, J. F.; Heller, M. The arginine-nitric oxide pathway: a target for new drugs. *Med. Res. Rev.* **1994**, *14*, 23–74.
(5) Schuman, E. M.; Madison, D. V. A requirement for the intercellular messenger nitric oxide in long term potentiation. *Science* **1991**, *254*, 1503–1506.
(6) Nathan, C. F.; Hibbs, J., Jr. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **1991**, *3*, 65–70.
(7) Lancaster, J., Jr.; Hibbs, J., Jr. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1223–1227.
(8) Brüne, B.; Lapetina, E. G. Activation of a cytosolic ADP-ribosyltransferase by nitric oxide-generating agents. *J. Biol. Chem.* **1989**, *264*, 8455–8458.
(9) Dimmeler, S.; Lottspeich, F.; Brüne, B. Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **1992**, *267*, 16771–16774.
(10) Molina y Vedia, L.; McDonald, B.; Reep, B.; Brüne, B.; Di Silvio, M.; Billiar, T. R.; Lapetina, E. G. Nitric oxide-induced *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.* **1992**, *267*, 24929–24932.
(11) Zhang, J.; Snyder, S. H. Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 9382–9385.
(12) McDonald, L. J.; Moss, J. Stimulation by nitric oxide of an NAD linkage to glyceraldehyde-3-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6238–6241.
(13) Kilbourn, R. G.; Jubran, A.; Gross, S. S.; Griffith, O. W.; Levi, R.; Adams, J.; Lodato, R. F. Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1132–1138.
(14) Petros, A.; Bennett, D.; Vallance, P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* **1991**, 1557–1558.
(15) Klabunde, R. E.; Ritger, R. C.; Helgren, M. C. Cardiovascular actions of inhibitors of endothelium-derived relaxing factor (nitric oxide) formation/release in anesthetized dogs. *Eur. J. Pharmacol.* **1991**, *199*, 51–59.
(16) Mulligan, M. S.; Hevel, J. M.; Marletta, M. A.; Ward, P. A. Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6338–6342.
(17) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620–1624.
(18) Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6368–6371.
(19) Rajfer, J.; Aronson, W. J.; Bush, P. A.; Dorey, F. J.; Ignarro, L. J. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N. Engl. J. Med.* **1992**, *326*, 90–94.
(20) Burnett, A. L.; Lowenstein, C. J.; Bredt, D. S.; Chang, T. S. K.; Snyder, S. H. Nitric oxide: a physiologic mediator of penile erection. *Science* **1992**, *257*, 401–403.
(21) Roberts, J. D.; Polaner, D. M.; Lang, P.; Zapol, W. M. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* **1992**, 818–819.
(22) Rossaint, R.; Falke, K. J.; López, F.; Slama, K.; Pison, U.; Zapol, W. M. Inhaled nitric oxide for the adult respiratory distress syndrome. *N. Engl. J. Med.* **1993**, *328*, 399–405.
(23) Ialenti, A.; Moncada, S.; Di Rosa, M. Modulation of adjuvant arthritis by endogenous nitric oxide. *Br. J. Pharmacol.* **1993**, *110*, 6338–6342.
(24) Middleton, S. J.; Shorthouse, M.; Hunter, J. O. Increased nitric oxide synthesis in ulcerative colitis. *Lancet* **1993**, 465–466.
(25) Kolesnikov, Y. A.; Pick, C. G.; Pasternak, G. W. N^G-Nitro-L-arginine prevents morphine tolerance. *Eur. J. Pharmacol.* **1992**, *221*, 399–400.
(26) Kolesnikov, Y. A.; Pick, C. G.; Ciszewska, G.; Pasternak, G. W. Blockage of tolerance to morphine but not to kappa opioids by a nitric oxide synthase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5162–5166.
(27) Peunova, N.; Enikolopov, G. Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. *Nature* **1993**, *364*, 450–453.
(28) Bredt, D. S.; Snyder, S. H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 682–685.
(29) Mayer, B.; John, M.; Bohme, E. Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS* **1990**, *277*, 215–219.
(30) Schmidt, H. H. H. W.; Pollack, J. S.; Nakane, M.; Gorsky, L. D.; Forstermann, U.; Murad, F. Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 365–369.
(31) Pollock, J. S.; Forstermann, U.; Mitchell, J. A.; Warner, T. D.; Schmidt, H. H. W.; Nakane, M.; Murad, F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10480–10484.
(32) Lamas, S.; Marsden, P. A.; Li, G. K.; Tempst, P.; Michel, T. Endothelial nitric oxide synthase: Molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6348–6352.
(33) Pollock, J. S.; Klinghofer, V.; Forstermann, U.; Murad, F. Endothelial nitric oxide synthase is myristylated. *FEBS* **1992**, *309*, 402–404.
(34) Busconi, L.; Michel, T. Endothelial nitric oxide synthase. N-Terminal myristoylation determines subcellular localization. *J. Biol. Chem.* **1993**, *268*, 8410–8413.

(35) Hevel, J. M.; White, K. A.; Marletta, M. A. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* **1991**, *266*, 22789–22791.
(36) Stuehr, D. J.; Cho, H. J.; Kwon, N. S.; Weise, M. F.; Nathan, C. F. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7773–7777.
(37) Janssens, S. P.; Shimouchi, A.; Quertermous, T.; Bloch, D. B.; Bloch, K. D. Cloning and expression of a cDNA encoding human endothelium derived relaxing factor/nitric oxide synthase. *J. Biol. Chem.* **1992**, *267*, 14519–14522.
(38) Wood, E. R.; Berger, H.; Sherman, P. A.; Lapetina, E. G. Hepatocytes and macrophages express an identical cytokine inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 767–774.
(39) Geller, D. A.; Lowenstein, C. J.; Shapiro, R. A.; Nussler, A. K.; Di Silvio, M.; Wang, S. C.; Nakayama, D. K.; Simmons, R. L.; Snyder, S. H.; Billiar, T. R. Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3491–3495.
(40) Sherman, P. A.; Laubach, V. E.; Reep, B. R.; Wood, E. R. Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. *Biochemistry* **1993**, *32*, 11600–11605.
(41) Ogura, T.; Yokoyama, T.; Fujisawa, H.; Kurashima, Y.; Esumi, H. Structural diversity of neuronal nitric oxide synthase mRNA in the nervous system. *Biochem. Biophys. Res. Commun.* **1993**, 1014–1022.
(42) Bredt, D. S.; Hwang, P. M.; Glatt, C. E.; Lowenstein, C.; Reed, R. R.; Snyder, S. H. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature (London)* **1991**, *351*, 714–718.
(43) White, K. A.; Marletta, M. A. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* **1992**, *31*, 6627–6631.
(44) Stuehr, D. J.; Ikeda-Saito, M. Spectral Characterization of brain and macrophage nitric oxide synthases. *J. Biol. Chem.* **1992**, *267*, 20547–20550.
(45) McMillan, K.; Bredt, D. S.; Hirsch, D. J.; Snyder, S. H.; Clark, J. E.; Masters, B. S. S. Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11141–11145.
(46) Nelson, D. R.; Kamataki, T.; Waxman, D. J.; Guengerich, F. P.; Estabrook, R. W.; Feyereisen, R.; Gonzalez, F. J.; Coon, M. J.; Gunsalus, I. C.; Gotoh, O.; Okuda, K.; Nebert, D. W. The P450 superfamily: update on new sequences, gene mapping, acvession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **1993**, *12*, 1–51.
(47) Narhi, L. O.; Fulco, A. J. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **1986**, *261*, 7160–7169.
(48) Tayeh, M. A.; Marletta, M. A. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.* **1989**, *264*, 19654–19658.
(49) Kwon, N. S.; Nathan, C. F.; Stuehr, D. J. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* **1989**, *264*, 20496–20501.
(50) Mayer, B.; John, M.; Heinzel, B.; Werner, E. R.; Wachter, H.; Schultz, G.; Bohme, E. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxidoreductase. *FEBS Lett.* **1991**, *288*, 187–191.
(51) Giovanelli, J.; Campos, K. L.; Kaufman, S. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7091–7095.
(52) Schmidt, H. H. H. W.; Smith, R. M.; Nakane, M.; Murad, F. Ca^{2+}/calmodulin-dependent NO synthase type I: a biopteroflavoprotein with Ca^{2+}/calmodulin-independent diaphorase and reductase activities. *Biochemistry* **1992**, *32*, 3243–3255.
(53) Hevel, J. M.; Marletta, M. A. Macrophage nitric oxide synthase: Relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry* **1992**, *31*, 7160–7165.
(54) Hevel, J. M.; Marletta, M. A. Influence of tetrahydrobiopterin on the structure of macrophage nitric oxide synthase. *Biochemistry*, submitted for publication.
(55) Hevel, J. M.; White, K. A.; Marletta, M. A. Purification of the inducible murine macrophage nitric oxide synthase: identification as a flavoprotein and detection of enzyme-bound tetrahydrobiopterin. In *The biology of nitric oxide. 2 Enzymology, biochemistry and immunology*; Moncada, S., Marletta, M. A., Hibbs, J. B., Jr., Higgs, E. A., Eds.; Portland Press: London, 1992; pp 19–21.
(56) Heinzel, B.; John, M.; Klatt, P.; Bohme, E.; Mayer, B. Ca2+/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem. J.* **1992**, 627–630.
(57) Baek, K. W.; Thiel, B. A.; Lucas, S.; Stuehr, D. J. Macrophage nitric oxide synthase. Purification, characterization, and role of the prosthetic groups and substrate in reglating their association into a dimeric enzyme. *J. Biol. Chem.* **1993**, *268*, 21120–21129.
(58) Stuehr, D. J.; Griffith, O. W. Mammalian nitric oxide synthases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1992**, *65*, 287–346.
(59) Stuehr, D. J.; Kwon, N. S.; Nathan, C. F.; Griffith, O. W.; Feldman, P. L.; Wiseman, J. N^{ω}-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* **1991**, *266*, 6259–6263.
(60) Pufahl, R. A.; Nanjappan, P. G.; Woodard, R. W.; Marletta, M. A. Mechanistic probes of N-hydroxylation of L-arginine by the inducible nitric oxide synthase from murine macrophages. *Biochemistry* **1992**, *31*, 6822–6828.
(61) Pufahl, R. A.; Marletta, M. A. Oxidation of N^{G}-Hydroxy-L-arginine by nitric oxide synthase: evidence for the involvement of the heme in catalysis. *Biochem. Biophys. Res. Commun.* **1993**, *193*, 963–970.
(62) Clement, B.; Jung, F.; Pfunder, H. *N*-hydroxylation of benzamidine to benzamidoxime by a reconstituted cytochrome P-450 oxidase system from rabbit liver: involvement of cytochrome P-450 IIC3. *Mol. Pharmacol.* **1993**, *43*, 335–342.
(63) Akhtar, M.; Calder, M. R.; Corina, D. L.; Wright, J. N. Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochem. J.* **1982**, *201*, 569–580.
(64) Stevenson, D. E.; Wright, J. N.; Akhtar, M. Mechanistic consideration of P-450 dependent enzymic reactions: studies on oestriol biosynthesis. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2043–2052.
(65) Fukuto, J. M.; Stuehr, D. J.; Feldman, P. L.; Bova, M. P.; Wong, P. Peracid oxidation of an *N*-hydroxyguanidine compound: a chemical model for the oxidation of N^{ω}-hydroxy-L-arginine by nitric oxide synthase. *J. Med. Chem.* **1993**, *36*, 2666–2670.
(66) Iyengar, R.; Stuehr, D. J.; Marletta, M. A. Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6369–6373.
(67) Olken, N. M.; Marletta, M. A. N^{G}-Methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry* **1993**, *32*, 9677–9685.
(68) Hibbs, J. B. J.; Vavrin, Z.; Taintor, R. R. L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* **1987**, *138*, 550–565.
(69) Stuehr, D. J.; Marletta, M. A. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7738–7742.
(70) Stuehr, D. J.; Marletta, M. A. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. *J. Immunol.* **1987**, *139*, 518–525.
(71) Hibbs, J. B. J.; Taintor, R. R.; Vavrin, Z. *Macrophage* cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **1987**, *235*, 473–476.
(72) Stuehr, D. J.; Marletta, M. A. Synthesis of nitrite and nitrate in murine macrophage cell lines. *Cancer Res.* **1987**, *47*, 5590–5594.
(73) Marletta, M. A.; Yoon, P. S.; Iyengar, R.; Leaf, C. D.; Wishnok, J. S. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **1988**, *27*, 8706–8711.
(74) Gross, S. S.; Stuehr, D. J.; Aisaka, K.; Jaffe, E. A.; Levi, R.; Griffith, O. W. Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by N^{G}-aminoarginine, N^{G}-nitroarginine and N^{G}-methylarginine. *Biochem. Biophys. Res. Commun.* **1990**, *170*, 96–103.
(75) Schmidt, K.; Klatt, P.; Mayer, B. Characterization of endothelial cell amino acid transport systems involved in the actions of nitric oxide synthase inhibitors. *Mol. Pharmacol.* **1993**, *44*, 615–621.
(76) Lambert, L. E.; Whitten, J. P.; Baron, B. M.; Cheng, H. C.; Doherty, N. S.; McDonald, I. A. Nitric oxide synthesis in the CNS endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci.* **1991**, *48*, 69–75.
(77) Lambert, L. E.; French, J. F.; Whitten, J. P.; Baron, B. M.; McDonald, I. A. Characterization of cell selectivity of two novel inhibitors of nitric oxide synthesis. *Eur. J. Pharmacol.* **1992**, *216*, 131–134.
(78) Olken, N. M.; Marletta, M. A. N^{G}-Allyl- and N^{G}-Cyclopropyl-L-arginine: Two novel inhibitors of macrophage nitric oxide synthase. *J. Med. Chem.* **1992**, *35*, 1137–1144.
(79) Rees, D. D.; Palmer, R. M.; Schulz, R.; Hodson, H. F.; Moncada, S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.* **1990**, *101*, 746–752.
(80) Olken, N. M.; Rusche, K. M.; Richards, M. K.; Marletta, M. A. Inactivation of macrophage nitric oxide synthase activity by N^{G}-methyl-L-arginine. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 828–833.
(81) Feldman, P. L.; Griffith, O. W.; Hong, H.; Stuehr, D. J. Irreversible inactivation of macrophage and brain nitric oxide synthase by L-N^{G}-methylarginine requires NADPH-dependent hydroxylation. *J. Med. Chem.* **1993**, *36*, 491–496.
(82) Klatt, P.; Schmidt, K.; Brunner, F.; Mayer, B. Inhibitors of brain nitric oxide synthase: bindinf kinetics, metabolism, and enzyme inactivation. *J. Biol. Chem.* **1994**, *269*, 1674–1680.
(83) Dwyer, M. A.; Bredt, D. S.; Snyder, S. H. Nitric oxide synthase: irreversible inhibition by L-N^{G}-nitroarginine in brain in vitro and in vivo. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 1136–1141.

(84) McCall, T. B.; Fellisch, M.; Palmer, R. M.; Moncada, S. Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.* **1991**, *102*, 234–238.
(85) Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. Selective inhibition of constitutive nitric synthase by N^G-nitroarginine. *Biochemistry* **1993**, *32*, 8512–8517.
(86) Misko, T. P.; Moore, W. M.; Kasten, T. P.; Nickols, G. A.; Corbett, J. A.; Tilton, R. G.; McDaniel, M. L.; Williamson, J. R.; Currie, M. G. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.* **1993**, *233*, 119–125.
(87) Wolff, D. J.; Datto, G. A.; Samatovicz, R. A.; Tempsick, R. A. Calmodulin-dependent nitric-oxide synthase. Mechanism of inhibition by imidazole and phenylimidazoles. *J. Biol. Chem.* **1993**, *268*, 9425–9429.
(88) Wolff, D. J.; Datto, G. A.; Samatovicz, R. A. The dual mode of inhibition of calmodulin-dependent nitric oxide synthase by antifungal imidazole agents. *J. Biol. Chem.* **1993**, *268*, 9430–9436.
(89) McMillan, K.; Masters, B. S. S. Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interactions. *Biochemistry* **1993**, *32*, 9875–9880.
(90) Moore, P. K.; Babbedge, R. C.; Wallace, P.; Gaffen, Z. A.; Hart, S. L. 7-Nitroindazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br. J. Pharmacol.* **1993**, *108*, 296–297.
(91) Rogers, N. E.; Ignarro, L. J. Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 242–249.
(92) O'Keeffe, D. H.; Ebel, R. E.; Peterson, J. A. Studies of the oxygen binding site of cytochrom P-450. Nitric oxide as a spin-label probe. *J. Biol. Chem.* **1978**, *253*, 3509–3516.
(93) Traylor, T. G.; Sharma, V. S. Why NO? *Biochemistry* **1992**, *31*, 2847–2849.
(94) Doyle, M. P.; Hoekstra, J. W. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. Inorg. Biochem.* **1981**, *14*, 351–8.
(95) Gross, S. S.; Levi, R. Tetrahydrobiopterin synthesis. an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J. Biol. Chem.* **1992**, *267*, 25722–25729.
(96) Hattori, Y.; Gross, S. S. GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: characterization, sequence and relationship to nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 435–441.
(97) Lyons, C. R.; Orloff, G. J.; Cunningham, J. M. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.* **1992**, *267*, 6370–6374.
(98) Xie, Q.-W.; Cho, H. J.; Calaycay, J.; Mumford, R. A.; Swiderek, K. M.; Lee, T. D.; Ding, A.; Troso, T.; Nathan, C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **1992**, *256*, 225–228.
(99) Lowenstein, C. J.; Glatt, C. S.; Bredt, D. S.; Snyder, S. H. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6711–6715.
(100) Cho, H. J.; Xie, Q.-W.; Calaycay, J.; Mumford, R. A.; Swiderek, K. M.; Lee, T. D.; Nathan, C. Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* **1992**, *176*, 599–604.
(101) Evans, T.; Carpenter, A.; Cohen, J. Purification of a distinctive form of endotoxin-induced nitric oxide synthase from rat liver. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5361–5365.
(102) Vorherr, T.; Knopfel, L.; Hofmann, F.; Mollner, S.; Pfeuffer, T.; Carafoli, E. The calmodulin binding domain of nitric oxide synthase and adenylyl cyclase. *Biochemistry* **1993**, *32*, 6081–6088.
(103) Zhang, M.; Vogel, H. J. Characterization of the calmodulin-binding domain of rat cerebellar nitric oxide synthase. *J. Biol. Chem.* **1994**, *269*, 981–985.
(104) Radomski, M. W.; Palmer, R. M. J.; Moncada, S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular cells. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 10043–10047.
(105) Huang, P. L.; Dawson, T. M.; Bredt, D. S.; Snyder, S. H.; Fishman, M. C. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **1993**, *75*, 1273–1286.