Irreversible Inactivation of Macrophage and Brain Nitric Oxide Synthase by L-N^G-Methylarginine Requires NADPH-Dependent Hydroxylation

Paul L. Feldman*

Glaxo Inc. Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709

Owen W. Griffith

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Hui Hong and Dennis J. Stuehr†

Department of Immunology, Cleveland Clinic, Cleveland, Ohio 44195, and Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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L-N^G-Methylarginine (NMA) is an established mechanism-based inactivator of murine macrophage nitric oxide synthase (mNOS). In this report, NMA is shown to irreversibly inhibit both mNOS (k_{inact} = 0.08 min^{-1}) and the recombinant constitutive brain NOS (bNOS). For both NOS isoforms, metabolism of NMA parallels that of the natural substrate L-arginine (ARG), in that it undergoes a regiospecific, NADPH-dependent hydroxylation to form L-N^G-hydroxy-N^G-methylarginine (NOHNMA). This intermediate then undergoes further NADPH-dependent oxidation to form L-citrulline (CIT). Authentic NOHNMA, synthesized from L-ornithine, irreversibly inhibited both mNOS (k_{inact} = 0.10 min^{-1}) and bNOS in an NADPH-dependent reaction. The conversion of either NMA or NOHNMA to CIT correlated with irreversible enzyme inactivation. Thus, the data suggest that enzyme inhibition occurs as a consequence of oxidative metabolism of the intermediate, NOHNMA. A unified mechanism is proposed that accounts for NO biosynthesis from ARG, for the inactivation of NOS by NMA and for the intermediacy of hydroxylated ARG or NMA derivatives in these processes.

Introduction

In recent years it has been demonstrated that nitric oxide (NO) is an important and ubiquitous effector molecule that plays a significant role in the regulation of a diverse set of mammalian physiological processes.[1] For example, NO generated from endothelial cells is known to play a critical role in regulating vascular resistance and platelet aggregation via activation of soluble guanylyl cyclase. In activated macrophages, NO acts as a cytostatic and cytotoxic agent by binding to catalytically essential non-heme iron present in enzymes such as ribonucleotide reductase[2] and the iron–sulfur enzymes of the mitochondrial electron transport pathway.[3] Also, NO is now known to be an important messenger in the brain and peripheral nervous system.[1a]

The biological action demonstrated by NO in a particular tissue is critically dependent upon the flux of NO reaching the targeted cells. Therefore, selective control of NO flux via regulation of its synthesis may have therapeutic implications.[4] In mammals, NO is generated by at least two distinct classes of nitric oxide synthase (NOS) enzymes.[1c,5] The constitutive NOS isoforms found in vascular endothelium, brain, and platelets, are Ca^{2+}/calmodulin-dependent enzymes that rapidly generate NO in response to intracellular calcium influx. Inducible NOS, found in cytokine or endotoxin stimulated macrophages, neutrophils, and hepatocytes, is a Ca^{2+}/calmodulin-independent enzyme that generates NO over an extended period of time (approx. 72 h) after its expression.

Several arginine-based irreversible inactivators of the NOS isoforms have been recently described.[1c,6] The inhibitor that has been studied most frequently is L-N^G-methylarginine (NMA)[3], a compound originally identified as a reversible inhibitor that binds competitively with L-arginine (ARG).[3,7] Further studies demonstrated that in the absence of ARG, NMA causes a relatively slow but irreversible inactivation of murine macrophage NOS (mNOS); irreversible inactivation is slowed further in the presence of ARG.[6a] The observation that inactivation by NMA is NADPH-dependent[6a] suggested that NMA might require metabolism to yield a reactive, inactivating species.

We have previously reported that the enzymatic conversion of ARG to NO and L-citrulline (CIT) by mNOS requires an initial NADPH-dependent N-hydroxylation to form L-N^G-hydroxyarginine (NOHARG).[8] NOHARG then undergoes further NADPH-dependent oxidation to generate CIT and the free radical NO (Scheme I). Reasoning that metabolism of NMA might occur through a similar pathway and lead to irreversible inhibition, we synthesized the putative intermediate, L-N^G-hydroxy-N^G-methylarginine (NOHNMA) and characterized its inhibition of both mNOS and constitutive brain (bNOS)[5] isoforms. Additionally, we show that irreversible inhibition of either NOS isoform by NMA depends on its regiospecific N^G-hydroxylation to form NOHNMA as an intermediate. Further NADPH-dependent metablism of NOHNMA to CIT leads to irreversible inactivation of either NOS isoform.

Chemistry

The synthesis of NOHNMA (1) follows closely to the route we described to synthesize L-N^G-hydroxyarginine (NOHARG).[9] Reaction of the known L-ornithine derivative 2 with N-methyl-O-benzylhydroxylamine[10] at 0 °C

† Case Western Reserve University.

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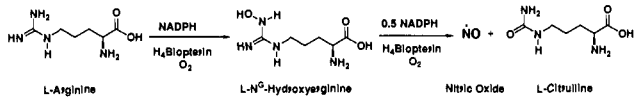
Scheme I. Enzymatic Conversion of L-Arginine to L-Citrulline and NO by NO Synthase



in CH_3CN in the presence of silver nitrate and triethylamine yielded **3** in 78% yield. Reduction of **3** with H_2 (1 atm) using 10% Pd–C as the catalyst in methanol removed the *O*-benzyl protecting group to yield **4** in 98%. Treatment of **4** with 4 N HCl/dioxane followed by reverse-phase HPLC purification produced NOHNMA bistrifluoroacetate salt as a hygroscopic oil in 23% yield. See Scheme II.

Results

Irreversible Inhibition of NOS Isoforms by NMA and NOHNMA. Our initial experiments characterized the irreversible inhibition of mNOS and bNOS by authentic NMA and NOHNMA. When incubations were carried out in the presence of NADPH (1 mM), both isoforms were irreversibly inhibited by NMA and NOHNMA in a time-dependent manner (Figure 2A and B). Maximal rates of inhibition with either compound were observed at concentrations at or exceeding 10 μM. For mNOS the k_{inact} with NOHNMA (0.1 min^{-1}) was greater than that for NMA (0.08 min^{-1}).[11]

When $NADP^+$ was substituted for NADPH in incubations containing either ARG, NMA, or NOHNMA, and either NOS isoform (Figure 2C and D), NMA, or NOHNMA no longer caused irreversible inhibition. Thus, irreversible inhibition of mNOS and bNOS by NMA or NOHNMA was NADPH-dependent.

Metabolism of NMA and NOHNMA Corresponds with Irreversible Inhibition. The above results suggested that an NADPH-dependent metabolism of NMA and NOHNMA was required to inactivate mNOS and bNOS. Our previous work had shown that the physiological substrate ARG is first converted to NOHARG via an NADPH-dependent hydroxylation and then undergoes a second NADPH-dependent, three-electron oxidation to generate CIT and the free radical NO.[8] Due to the nonequivalence of NMA's terminal guanidino nitrogens, a similar metabolism of NMA could conceivably occur by either or both of two pathways (Scheme III). Enzymatic hydroxylation on the N^G-methyl guanidino nitrogen would lead to NOHNMA. If further oxidation of NOHNMA occurs exclusively on the hydroxylated nitrogen, as is the case with the normal intermediate NOHARG,[8] then the final expected products are CIT and the nitrosomethane

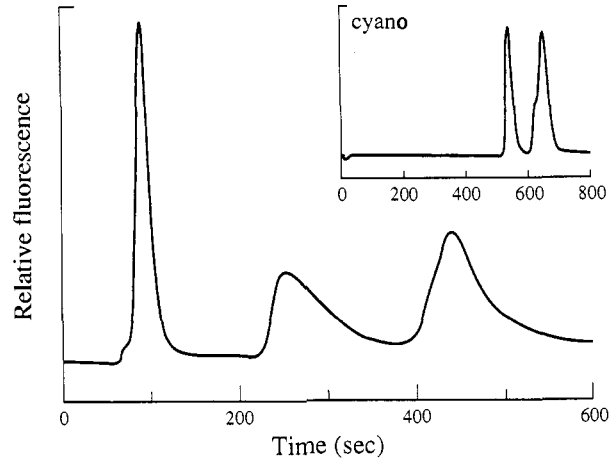


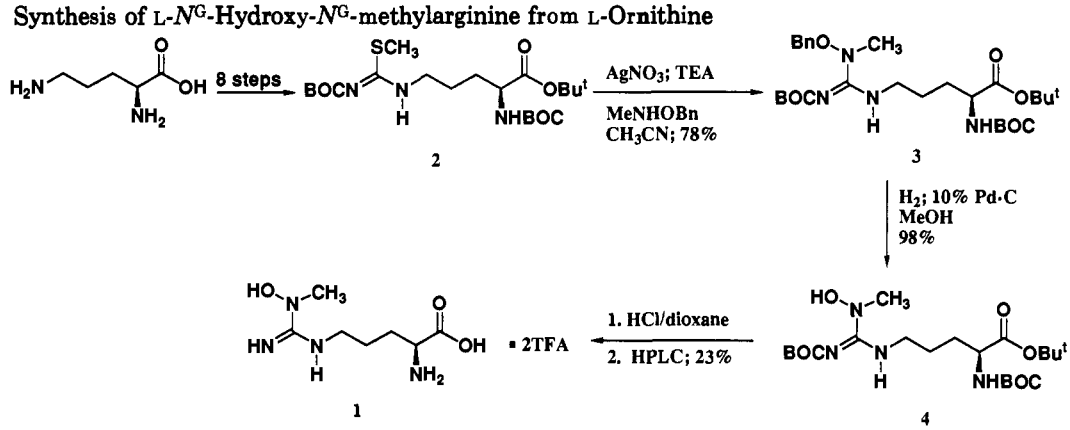
Figure 1. Reverse-phase HPLC separation of underivatized standard compounds (2 nmol each) and their retention times. Separation on a C18 column of CIT (94 s), NMA (253 s), and NOHNMA (444 s). Inset: Separation on a cyano reverse-phase column of CIT (541 s) and L-N^G-methylcitrulline (651 s) under otherwise identical conditions.

cation radical.[12] Alternatively, if the initial hydroxylation occurred on the non-methylated N^G-guanidino nitrogen, and the subsequent oxidation occurred at the hydroxylated nitrogen, this would yield L-N^G-methylcitrulline and NO (path b, Scheme III). To determine which nitrogen was enzymatically hydroxylated, we incubated [^{14}C]NMA[13] with either mNOS or bNOS in the presence of NADPH and analyzed the products by HPLC. Both enzymes produced two major radiolabeled metabolites which had retention times on C_{18} reverse-phase HPLC identical to authentic CIT and NOHNMA (Figure 3). The two metabolites were isolated and found to coelute with authentic CIT and NOHNMA standards on cyano reverse-phase HPLC (see Experimental Section). Furthermore, neither metabolite coeluted with an authentic sample of L-N^G-methylcitrulline[14] on cyano reverse-phase HPLC. Enzymatic production of NO (measured as accumulated NO_2^- and NO_3^-) was not observed. These data indicate that both isoforms hydroxylated NMA on the methyl-substituted nitrogen.

The ability of mNOS to convert NMA to NOHNMA and CIT was inhibited when the enzymatic incubations were carried out in the presence of the flavoprotein inhibitor diphenyleneiodonium,[15] a previously described irreversible inhibitor of the NOS isoforms (Figure 3, right panel). This suggests that the metabolism of NMA, like that of the natural substrate, ARG, involves the flavoprotein domain of NOS.

Time-course studies revealed that the enzymatic con-

Scheme II. Synthesis of L-N^G-Hydroxy-N^G-methylarginine from L-Ornithine



version of authentic NMA or NOHNMA to CIT corresponded with a cumulative, irreversible enzyme inactivation, as shown for mNOS (Figure 4). The amount of CIT synthesized from either NMA or NOHNMA at the time of 50% inactivation was similar (Figure 4) and represented approximately ≥200 catalytic turnovers per inhibitory event. Essentially identical results were observed with the bNOS isoform (data not shown).

Our attempts to determine the amount of specific NADPH oxidation required to generate CIT from either inhibitor were unsuccessful because both NMA and NOHNMA promote uncoupled NADPH oxidation by mNOS and bNOS (data not shown). The uncoupling of NADPH oxidation is associated with the generation of superoxide and hydrogen peroxide, a process that might contribute to the enzyme inactivation mediated by NMA or NOHNMA. However, the fact that added catalase (1000 units/mL) and superoxide dismutase (100 units/mL) did not affect the kinetics of NMA and NOHNMA irreversible inhibition of either NOS isoform (data not shown) argues against a significant role for these oxidants in the inactivation.

Our data indicated that the NOS isoforms were most likely inhibited during the second phase of the enzymatic reaction, i.e., during the NADPH-dependent oxidation of the intermediate NOHNMA to generate CIT and an unknown product that is distinct from NO. On the basis of the established metabolism of NOHARG,[8] it is probable that the radical cation of nitrosomethane results from NOS-mediated metabolism of NMA and NOHNMA (see below). We considered the possibility that the radical cation might be converted to neutral nitrosomethane by reductants on the enzyme or in solution. Nitrosomethane is a highly reactive gas, but rapidly tautomerizes in solution to form the relatively less reactive formaldoxime.[12,16] Incubation of either bNOS or mNOS with 0.1 to 1 mM formaldoxime did not lead to the inactivation of either isoform, suggesting that the oxime tautomer was not the inhibitory species.

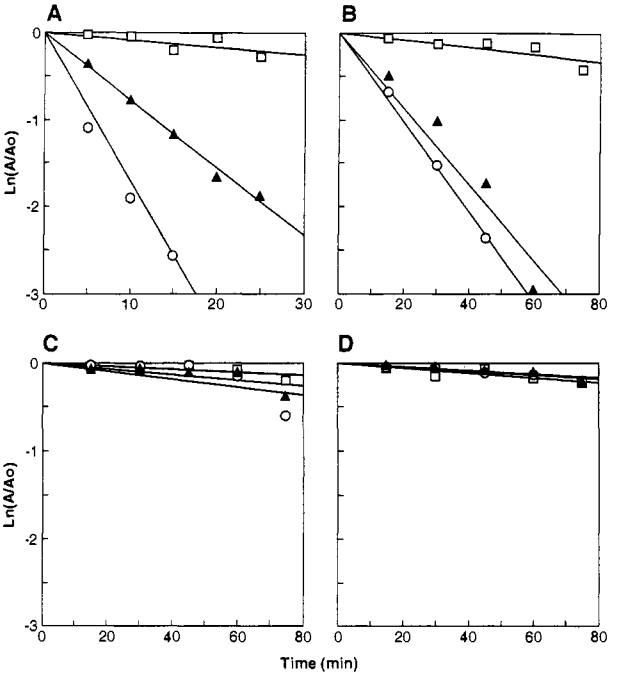
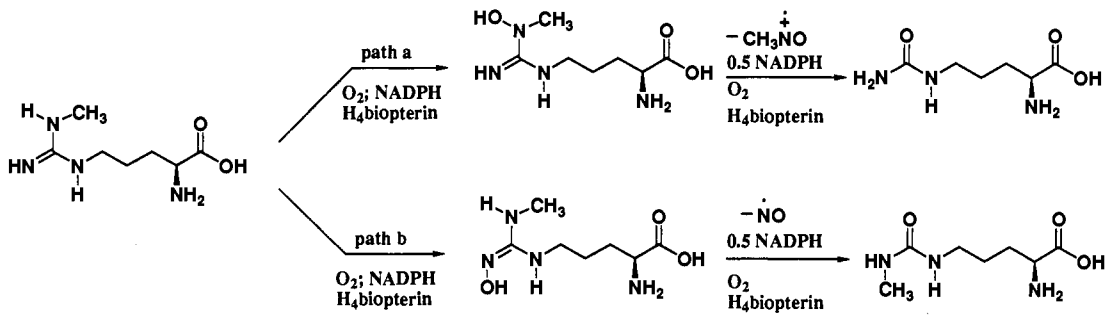


Figure 2. Time-dependent irreversible inactivation of bNOS and mNOS by NMA and NOHNMA. Purified bNOS (panel A) or mNOS (panel B) was incubated at 37 °C in the presence of 1 mM NADPH with either 1 mM ARG (bNOS) or no substrate (mNOS) (open squares), 10 μM NMA (solid triangles), or 10 μM NOHNMA (open circles) and assayed for residual NO synthase activity at the times indicated, as detailed in the Experimental Section. Points shown are the mean of three experiments. Purified bNOS (panel C) and mNOS (panel D) were incubated with either 1 mM ARG (bNOS) or no substrate (mNOS), 10 μM NMA, or NOHNMA as described in caption above, except 1 mM NADPH was replaced 1 mM $NADP^+$. Points shown are the mean of two experiments.

Discussion

Recent advances in the biochemical and molecular biological characterization of the NOS isoforms have significantly increased our knowledge of NO biosynthesis.[1c] Both the macrophage and brain isoforms are homodimers (mNOS monomer MW = 130 kDa[17], bNOS monomer MW = 150 kDa[18]), catalyze the oxygen- and NADPH-dependent five-electron oxidation of ARG to CIT and NO, require tetrahydrobiopterin,[19] and contain one flavin adenine dinucleotide (FAD) and one flavin mononucleotide (FMN) per subunit.[5,17] This last characteristic is noteworthy in that just one other mammalian flavoprotein (NADPH-cytochrome P-450 reductase) and two bacterial flavoproteins (sulfite reductase, cytochrome $P\text{-}450_{BM\text{-}3}$) are known to contain both FAD and FMN.[20] Gene sequence analysis of the NOS isoforms and of NADPH-cytochrome P-450 reductase show that all share a "reductase" region which encodes for putative NADPH, FAD, and FMN binding sites.[5] These findings, in combination with the present results, have important implications regarding the catalytic mechanism of NOS. On the basis of studies with cytochrome P-450 reductase,[21] it appears likely that the NOS flavins transfer electrons from NADPH to an electron acceptor within the enzyme and that the acceptor then catalyzes the oxygenation reaction(s) associated with NO synthesis.[1c,5] It has been demonstrated that the two electron oxidation of ARG to NOHARG is coupled to the oxidation of one molecule (two electrons) of NADPH.[8] This data coupled with the recent discovery that both mNOS and bNOS contain two heme (iron protoporphyrin IX) groups per homodimer[22] further supports the sug-

Scheme III. Potential Metabolic Routes for NMA



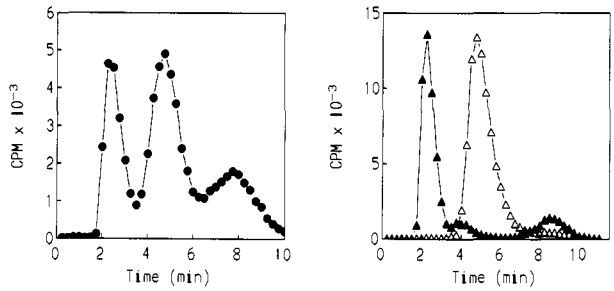


Figure 3. Metabolism of [^{14}C]NMA to NOHNMA and CIT by bNOS (left panel) and mNOS (right panel). One-hour incubations containing purified enzyme and [^{14}C]NMA were carried out and fractions were analyzed by scintillation counting as detailed in the Experimental Section. For bNOS (left panel), the chromatogram contained three peaks that displayed the retention times of authentic CIT (2.2 min), NMA (4.4 min), and NOHNMA (7.8 min). For mNOS (right panel), incubations were carried out in the presence (open triangles) or absence (solid triangles) of the NOS inhibitor diphenyleneiodonium (10 μM). The three peaks are CIT (2.4 min), NMA (5.3 min), and NOHNMA (8.5 min). Data shown is representative of three similar experiments.

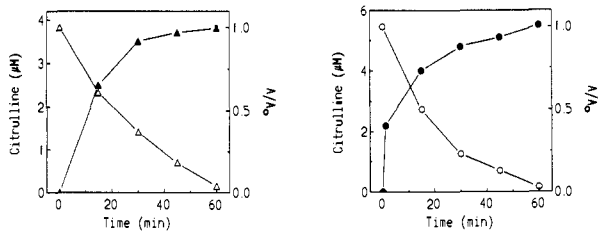


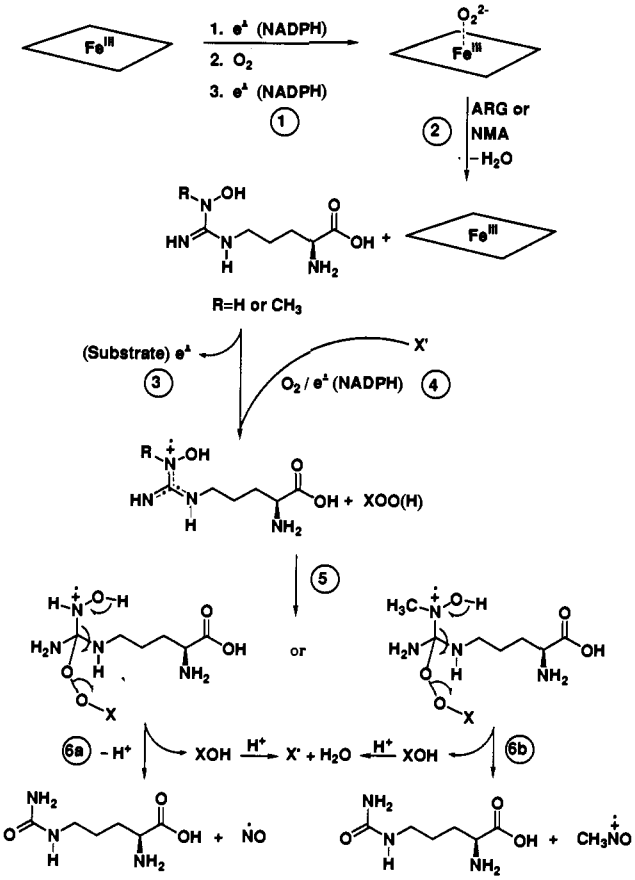
Figure 4. Conversion of NMA or NOHNMA to CIT by mNOS correlates with irreversible enzyme inactivation. Incubations contained NMA (25 μM; left panel) or NOHNMA (25 μM, right panel) and 5 μg purified mNOS and were run as detailed in the Experimental Section. At the times indicated, duplicate aliquots were removed to determine the fraction of NOS activity that remained (open triangles and circles) and for HPLC analysis (solid triangles and circles). HPLC aliquots were mixed with 10 μM diphenyleneiodonium to stop the enzymatic reaction. Data shown is representative of three experiments.

gestion that the initial NOS reaction is analogous to that of NADPH-cytochrome P-450 reductase (Scheme IV, steps 1 and 2). Indeed, enzymatic formation of NOHARG or NOHNMA is similar to the N-hydroxylation of 2,4,6-trimethylacetophenone imine to form the oxime, a reaction catalyzed by a microsomal cytochrome P-450.[23]

In contrast to the biologically precedented initial hydroxylation of ARG and NMA, the further three-electron oxidation of NOHARG and NOHNMA to CIT and a radical product is without obvious precedent. In Scheme IV, steps 4–6, a mechanism is depicted that is consistent with our previous observations that the conversion of NOHARG to CIT is tetrahydrobiopterin- and oxygen-dependent and requires only one electron from NADPH.[8] Since NADPH must donate two electrons, we again envision the flavins as mediating consecutive one-electron transfers from a two-electron donor; therefore, three molecules of NADPH would serve two full catalytic cycles.[1c] The scheme shown accommodates the parallel processing of ARG and NMA, thereby accounting for the formation of CIT from both compounds. Details of individual steps are presented in the legend of Scheme IV.

The irreversible inactivation of mNOS by NMA,[6a] L-N^G-allylarginine,[6b] L-N^G-aminoarginine,[1c] and L-N^E-(iminoethyl)ornithine[24] have been previously reported. Very recently, Pufahl et al. confirmed our observations establishing the intermediacy of NOHARG in the mNOS-mediated oxidation of ARG and noted that NOHNMA was also an irreversible inhibitor.[25] In the present studies we have demonstrated the regiospecific hydroxylation of NMA by both mNOS and bNOS and have shown that further metabolism of the intermediate NOHNMA to CIT is associated with irreversible enzyme inactivation. We note that nitrosomethane radical cation, the expected second product from NMA or NOHNMA oxidation, might inactivate NOS in a number of ways. Certain organic hydroxylamines are oxidized by cytochrome P-450 enzymes to nitroso compounds which form noncovalent but essentially irreversible complexes with the heme iron.[26] Since these enzyme–inhibitor complexes often display a red shift in the Soret band to give a maximum around 450 nM, we have looked for a similar shift in the Soret absorbance of NMA-inactivated mNOS; however, no shift

Scheme IV. Proposed Mechanism of Processing ARG and NMA by NO Synthase[a]



[a] The oxidation of ARG or NMA to NO or the radical cation of nitrosomethane is accounted for by the steps shown. Step 1 is a formal representation of the activation of dioxygen utilizing the model of cytochrome P-450 activation of dioxygen.[30] The combination of steps 1 and 2 lead to the now-established intermediates NOHARG[8] or NOHNMA (this work). Steps 3 and 4, representing one-electron donations by hydroxylated substrate and NADPH, respectively, produce the oxidizing species XOO(H). XOO(H) may be an oxy-heme species similar to that produced in step 1 or may be the peroxy derivative of tetrahydrobiopterin. X' may either be heme or dihydrobiopterin. Step 5 shows the nucleophilic attack of XOO(H) on the radical cation of NOHARG or NOHNMA; steps 6a and 6b show the further reaction to final products. Note the electron transfers shown in steps 6a and 6b constitute a two-electron oxidation of the hydroxylated nitrogen; the overall scheme thus accounts for a five-electron oxidation. Although NOHARG and NOHNMA are depicted as donating an electron in step 3, it is possible that oxidation occurs later in the scheme from a metabolite of the hydroxylated intermediates. In the extreme case, electron donation could be the last step converting nitroxyl (HNO) or nitrosomethane (CH_3NO) to NO or nitrosomethane radical cation, respectively.

was found (data not shown). It is also possible that nitrosomethane or its cation radical inactivate the enzymes through covalent modification of the heme or active site amino acid residues, as occurs with some P-450 inhibitors.[27] Finally, destabilization of NOS may occur during the metabolism of NOHNMA such that dissociation into inactive monomers occurs. These possibilities are currently being investigated to better understand the mechanism-based inhibition at the molecular level.

It is interesting that both mNOS and bNOS show nearly identical behavior with respect to NMA and NOHNMA inhibition. These results coupled with the 60% homology shared by both isoforms[5] and their similar biochemistry suggests that the mechanism by which both enzymes convert ARG to CIT and NO is very similar if not identical.

Summary

NMA and NOHNMA were shown to be mechanism-based irreversible inhibitors of both the inducible (mNOS) and a constitutive (bNOS) enzyme isoform. Inhibition of either isoform required that NMA be metabolized analogously to the natural substrate, ARG. NMA was initially hydroxylated at its methylated guanidino nitrogen to yield NOHNMA, which underwent a subsequent NADPH-coupled oxidation to generate CIT and an uncharacterized metabolite. The conversion of NOHNMA to CIT was associated with the irreversible enzyme inactivation. On the basis of a proposed mechanism for NO biosynthesis from ARG, the unknown metabolite of NOHNMA is postulated to be the radical cation of nitrosomethane or, if reduction occurs, nitrosomethane itself. Either species, or further metabolized reactive products, could represent the inactivating agent(s).

Experimental Section

Chemistry. ^{13}C NMR spectra were determined using a Varian spectrometer (300 MHZ superconducting, FT instrument), with $CDCl_3$ as the solvent, unless noted otherwise, and are expressed in ppm relative to the $CDCl_3$ central peak resonating at 77.0 ppm. Flash silica gel chromatography was conducted with 32–63 μm grade Universal Scientific grade silica gel. Concentration of reaction mixtures was performed by using a rotary evaporator at water aspirator pressure unless otherwise noted.

α-*N*-(*tert*-Butoxycarbonyl)-N^G-(benzyloxy)-N^G-methyl-$N^{G'}$-(*tert*-butoxycarbonyl)-L-arginine *tert*-Butyl Ester (3). To a solution of α-*N*-(*tert*-butoxycarbonyl)-δ-[N^1-(*tert*-butoxycarbonyl)-*S*-methyl)-1-isothioureido]-L-norvaline *tert*-butyl ester[9] (2) (639 mg, 1.39 mmol), triethylamine (0.24 mL, 1.74 mmol, 125 mol%), and *O*-benzyl-*N*-methylhydroxylamine[10] (285 mg, 2.08 mmol, 150 mol%) in CH_3CN (14 mL) at 0 °C was added a solution of $AgNO_3$ (295 mg, 1.74 mmol, 125 mol%) in CH_3CN (4 mL) dropwise. The reaction was stirred at 0 °C for 2 h then filtered through Celite. The filtrate was concentrated and the residue chromatographed on silica gel. An eluent mix of 3/2 EtOAc/hexanes was used to remove the nonpolar impurities followed by 85/15 $CHCl_3$/MeOH to obtain 3 as an oil: 595 mg; 78%; ^{13}C NMR δ 171.2, 159.6, 159.3, 155.0, 134.3, 129.1, 128.7, 128.4, 81.5, 79.3, 77.6, 75.9, 53.2, 41.9, 39.1, 29.8, 28.0, 27.64, 27.61, 24.9.

α-*N*-(*tert*-Butoxycarbonyl)-N^G-hydroxy-N^G-methyl-$N^{G'}$-(*tert*-butoxycarbonyl)-L-arginine *tert*-Butyl Ester (4). A mixture of 3 (516 mg, 0.94 mmol) and 10% Pd–C (75 mg) in MeOH (15 mL) was stirred under 1 atm of H_2 for 18 h. The reaction mixture was filtered through Celite and the filtrate concentrated using a cool water bath. The residue was chromatographed through a short plug of silica gel (4/1 $CHCl_3$/MeOH) and concentrated to give 4 as an oil: 425 mg, 98%; ^{13}C NMR δ 171.3, 155.2, 150.9, 150.3, 83.1, 81.9, 79.6, 53.2, 43.7, 40.7, 29.5, 28.0, 27.73, 27.67, 25.3.

L-N^G-Hydroxy-N^G-methylarginine Bistrifluoroacetate Salt (1). A solution of 4 (373 mg, 0.811 mmol) in HCl in dioxane (4 N, 10 mL) was stirred at 23 °C for 15 h. The solvent was removed and the residue purified by C18 (Vydac C18 5-mm preparatory column reverse-phase column) reverse-phase HPLC using H_2O/0.1% TFA (10 mL/min) as the eluent. The product was isolated by lyophylizing the frozen aqueous solution to give the product as a hygroscopic foam: 80 mg, 23%; mass spectrum, positive ion FAB in thioglycerol MH^+ calcd 205, found MH^+ 205; ^{13}C NMR (CD_3OD) δ 171.5, 159.5, 53.5, 42.2, 40.7, 28.5, 25.7.

Enzyme Purification. mNOS was isolated from the supernatants of RAW 264.7 cells that had been cultured for 12 h with 100 units/mL interferon-γ and 1 μg/mL bacterial lipopolysaccharide to induce expression of mNOS.[17a] bNOS was obtained from the supernatants of R29 human kidney cells that were stably transfected with an expression plasmid encoding bNOS.[5a] The purification of both enzymes was accomplished by chromatography on 2′,5′ ADP sepharose and Mono-Q (anion exchange) columns as described in detail elsewhere.[17a,22a]

Incubation of mNOS and bNOS with Inhibitors. The mNOS (5 μg) was incubated at 37 °C in 40 mM Hepes buffer, pH 7.6, containing 4 μM FAD, 4 μM H4biopterin, 3 mM DTT, and 0.5–50 μM NMA or NOHNMA. The bNOS (5 μg) was incubated under the same conditions except that the reactions also contained 0.83 mM Ca^{2+}, 10 μg/mL calmodulin, and 0.6 mM EDTA.[18] Reactions were initiated by adding NADPH (0.1–1 mM) and 30–50 μL aliquots were removed to assay enzymatic activity at various times. In the case of [^{14}C]NMA metabolism, [^{14}C]NMA (0.23 μCi/nmol) and unlabeled NMA were added such that the reactions were 14 μM in total NMA and contained 1–2 × 10^5 cpm. Prior to HPLC analysis, reactions were stopped by adding the irreversible NOS inhibitor diphenyleneiodonium (DPI) to 10 μM.[15]

Determination of Enzyme Activity. NO synthase activity was assayed at 37 °C using a modified spectrophotometric oxyhemoglobin method.[28] Aliquots (30–50 μL) were removed from the incubations at designated times and added to prewarmed cuvettes containing 40 mM Hepes buffer, pH 7.6, 5 mM L-arginine, 1 mM NADPH, 4 μM FAD, 4 μM H_4biopterin, 0.15 mM DTT, 10 units/mL superoxide dismutase, 100 units/mL catalase, and 5–10 μM oxyhemoglobin. Final volume was 700 μL. The relative rates of NO synthesis were determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm.[28] A reference cuvette containing all constituents minus enzyme was run simultaneously with each sample to control for the background oxidation of oxyhemoglobin. Rates obtained at 0 min were considered maximal and used to calculate the fraction of activity remaining at each subsequent timepoint.

Detection and Quantitation of L-Citrulline, NOHArg, NOHNMA, and *N*-Methyl-L-citrulline. Protein was removed from enzyme incubations using spin-X filtration devices prior to analysis. Amino acids were separated and detected using a Waters HPLC equipped with either a C18 or a cyano reverse-phase column and fluorometric detection. Underivatized samples were injected onto the columns and eluted with an aqueous solution of 17 mM L-proline and 8 mM cupric acetate, pH 6.8, at 0.5 mL/min. Amino acids in the effluent were detected fluorometrically after in-line postcolumn derivatization with an o-phthalaldehyde (OPA) reagent as described.[8] A typical HPLC separation of authentic CIT, L-N^G-methylcitrulline, NOHNMA, and NMA on the C18 and cyano reverse-phase columns is shown in Figure 1. Authentic NMA and NOHNMA displayed identical retention times on the cyano column (22.67 min, not shown). Although the exact retention times of standards were found to vary from batch to batch of the cupric–proline running buffer, the relative positions of the peaks were invariant. To quantitate metabolites arising from [^{14}C]NMA, column effluent was collected in 0.125 mL (15 s) fractions without undergoing in-line postcolumn derivatization with the OPA reagent, and the radioactivity contained in 0.1 mL of each fraction was determined using liquid scintillation counting. Postcolumn flow times were observed to increase proportionally in the absence of in-line postcolumn derivatization.

For studies with nonradiolabeled NMA and NOHNMA, amino acid products were quantitated by prederivatization with o-phthalaldehyde, isocratic HPLC separation on the C18 column, and fluorometric detection as described.[29] Peaks were quantitated using curves generated from authentic standards. Retention

times for authentic OPA-derivatized CIT, L-N^G-methylcitrulline, NMA, and NOHNMA were 19.3, 23.6, 26.9, 28.3 min, respectively.

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