OXIDATION OF N^G-HYDROXY-L-ARGININE BY NITRIC OXIDE SYNTHASE: EVIDENCE FOR THE INVOLVEMENT OF THE HEME IN CATALYSIS

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Summary The involvement of the protoporphyrin IX heme iron of macrophage nitric oxide synthase (NOS) in the oxidation of N^G-hydroxy-L-arginine (L-NHA) to nitric oxide (NO) and citrulline was investigated by carbon monoxide (CO) inhibition studies and binding difference spectroscopy. A CO:oxygen mixture (80:20) was found to inhibit the reaction by 33% with L-NHA as a substrate compared to 57% with L-arginine. Spectral perturbations were observed upon the addition of L-NHA to oxidized NOS, producing a type I binding difference spectrum with a maximum at 384 nm and minimum at 420 nm. In addition, L-NHA was incapable of reducing anaerobic oxidized NOS in the absence of NADPH. These studies support the involvement of the heme in the oxidation of L-NHA to NO and citrulline, indicating that the heme functions in both of the currently characterized oxidative steps of the NOS reaction.   © 1993 Academic Press, Inc.

Nitric oxide synthases (NOS's; EC 1.14.23) constitute an expanding family of unique enzymes responsible for the biosynthesis of nitric oxide (NO) in mammalian cells. NO has been shown over the past decade to be an important mediator in vasodilation, neurotransmission and cellular cytotoxicity (1, 2). This enzyme, although found in a number of diverse cell and tissue types, can be categorized into two distinct types: a constitutive Ca^{2+}/calmodulin regulated NOS such as those found in neurons (3, 4) and the endothelium (5) and a cytokine-inducible form found in macrophages (6, 7) not responsive to Ca^{2+}/calmodulin. Most studies have focused on the isoforms isolated from rat brain and murine macrophages and therefore are the best characterized to date. However, it appears that all NOS's are catalytically and structurally similar.

NO and citrulline are formed through the oxidation of one of the guanido nitrogens of L-arginine at the expense of NADPH and molecular oxygen (O_2) (Figure 1). N^G-hydroxy-L-arginine (L-NHA) has been observed as an intermediate in reaction resulting from the monooxygenation of L-arginine (8, 9). NOS contains one enzyme-bound FAD, FMN and

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; L-NHA, N^G-hydroxy-L-arginine; H_4B, (6R)-tetrahydro-L-biopterin; CO, carbon monoxide; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; NO_2^-, nitrite; NO_3^-, nitrate; PCD, protocatechuate 3,4-dioxygenase; PCA, protocatechuic acid; TCA, trichloroacetic acid.
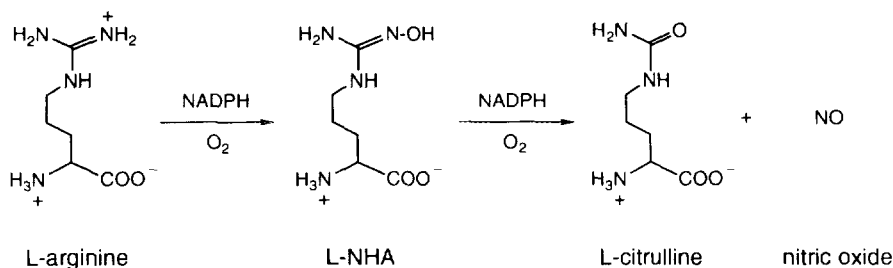
Figure 1. NOS catalyzed oxidation of L-arginine to NO and citrulline is shown above. L-NHA is produced by the N-hydroxylation of L-arginine.

protoporphyrin IX heme iron per subunit (10-12) and appears to function as a homodimer. A requirement for (6R)-tetrahydro-L-biopterin (H4B) has also been observed and at least with the macrophage isoform, one H4B per 130 kDa subunit has been found to be enzyme-bound (13), which yields enzyme with the highest activity. In addition, significant sequence homology exists between NADPH cytochrome P450 reductase and the carboxy terminus of NOS in the flavin and nucleotide binding domains (14, 15). The recent observation that NOS contains one heme iron, coupled with the earlier findings of two flavins, suggests that NOS is the first example of a mammalian catalytically self-sufficient P450 enzyme, containing both a heme and reductase domain contained within the same polypeptide chain.

The heme has been shown to be involved in catalysis when L-arginine is used as a substrate (10, 16). This was demonstrated through inhibition of enzymatic activity in the presence of carbon monoxide (CO). Herein, we report a number of findings that indicate that the heme is also involved in the oxidation of L-NHA to NO and citrulline with macrophage NOS.

Materials and Methods

Materials and General Methods
Murine macrophage NOS (from RAW 264.7 cells immunostimulated with *E. coli* lipopolysaccharide and recombinant mouse interferon-γ) was purified in the presence of H4B as previously described (13). L-Arginine, Hepes, DTT, NADPH and TCA were purchased from Sigma. H4B was purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and prepared in 15 mM Hepes (pH 7.5) containing 100 mM DTT. Mixtures of CO and O_2 were prepared with a Matheson gas mixer. UV-VIS spectroscopy was done with a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller set at 25° C. When necessary, all spectra were corrected for dilutions. L-NHA was synthesized as previously described and used as the hydrochloride salt (9). NO_2^- and NO_3^- levels were determined by an automated procedure as previously described (17). NO_2^- and NO_3^- standards (0 - 28 μM) contained an equivalent concentration of TCA as added to each sample. Protein concentrations were determined by the Bradford protein microassay (Bio-Rad) using bovine serum albumin as a standard (Boehringer Mannheim).

Difference Spectroscopy
Substrate-induced binding difference spectra were obtained as follows. Purified NOS (0.4 μM) in 15 mM Hepes (pH 7.5) was added to the sample cuvette and scanned from 350 to 475 nm against a reference cuvette containing buffer. L-NHA was then added to the sample cuvette (final concentration of 250 μM) and the resulting spectrum recorded from 350 to 475 nm. The difference spectrum was obtained by subtracting the first spectrum from the second.

CO Inhibition

Siliconized 2 mL microfuge tubes containing 15 mM Hepes (pH 7.5), L-arginine or L-NHA (400 μM), H$_4$B (50 μM), DTT (600 μM) and NOS (0.8-1.6 μg) were sealed with septa (total volume of 300 μL) and equilibrated with either a CO:O$_2$ gas mixture (80:20) or ambient air (as a control) for 8 minutes at room temperature. The reactions were then initiated with NADPH (100 μM) via a gas-tight syringe and incubated at 37° C for 5 minutes. Exposure to both the CO:O$_2$ mixture and air were stopped after the reactions were initiated to avoid the loss of NO through the vent. All assays were terminated by TCA (0.2 M final concentration) via addition by a gas-tight syringe and samples were subsequently analyzed for NO$_2^-$ and NO$_3^-$, the solution decomposition products of •NO, as described above. In some instances, assays were initiated with L-NHA instead of NADPH; in these cases, all other conditions were identical to those described above.

Anaerobic Reduction of Oxidized NOS by L-NHA

NOS (0.8 μM) in 100 mM Hepes (pH 7.5) and protocatechuate 3,4-dioxygenase (PCD) (475 U; 1 U is the amount of enzyme that catalyzes the disappearance of 1 μmole substrate min^{-1}) were added to a cuvette, sealed with a septum and equilibrated with CO for 10 minutes at room temperature. The sample was then placed in the spectrophotometer and scanned from 250 to 600 nm; the reference cuvette contained only 100 mM Hepes (pH 7.5). Anaerobic protocatechuic acid (PCA) was added via a gas-tight syringe to a final concentration of 200 μM. After 5 minutes, anaerobically prepared L-NHA was added via a gas-tight syringe (final concentration 500 μM) and spectra were subsequently recorded every 2 minutes for 12 minutes. Anaerobic NADPH (20 μM final concentration) was then added by gas-tight syringe to the sample cuvette and the resulting spectrum was recorded. All anaerobic solutions were prepared by purging extensively with argon.

Results

Binding Spectrum Produced by L-NHA

The absorbance spectrum of NOS displayed a broad Soret band typically centered around 395-400 nm, which is characteristic of a high-spin heme. The addition of L-NHA (final concentration 250 μM) to this enzyme caused a slight blue-shift of the Soret band. The resulting difference spectrum shows an increase at 384 nm and a decrease at 420 nm (Figure 2). The magnitude of the absorbance change (from peak to trough) was dependent on the concentration of L-NHA added (data not shown).
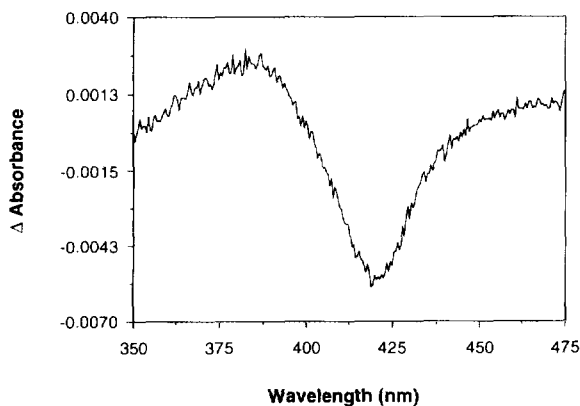

Figure 2. Type I difference spectrum produced by the addition of L-NHA (250 μM) to oxidized NOS (0.4 μM) in 15 mM Hepes (pH 7.5).

CO Inhibition of NO Formation from L-NHA

Several experiments were carried out to determine if CO would serve as an inhibitor when L-NHA was used as a substrate. As shown in Table 1, equilibration of the reaction mixture with an 80:20 mixture of $CO:O_2$ resulted in approximately 33% inhibition of product formation with respect to assays done under an atmosphere of air. If the reaction was initiated by the addition of L-NHA instead of NADPH as above, the degree of CO inhibition was nearly unchanged at 37% (36.8 ± 4.5%, n=3, data not shown). Inhibition of NO_2^- / NO_3^- formation was also examined under identical experimental conditions with L-arginine as the substrate. In this case, the amount of inhibition caused by CO (57%) was approximately 1.75 times that observed with L-NHA. All assays contained saturating concentrations of substrates and NADPH; reactions could then be stopped at the desired time by the addition of TCA. The formation of NO_2^- / NO_3^- during the 5-minute assay period was linear (not shown) and a $CO:O_2$ mixture of 80:20 produced maximal inhibition (not shown).

Reaction of Oxidized NOS with L-NHA

Recently published enzymatic mechanisms of NO formation from L-NHA (10, 11, 18) have suggested that L-NHA may provide one electron to the heme iron during turnover. Reduced NOS (ferrous) under a CO atmosphere forms a high extinction coefficient P450 CO-ferrous iron complex at 446 nm and this was used to determine if L-NHA was capable of reducing oxidized NOS. Oxidized (ferric) NOS was made anaerobic and placed under a CO atmosphere followed by the addition of anaerobically prepared L-NHA (500 μM final concentration). PCD/PCA was used as an oxygen-scavenging system. If L-NHA were to reduce the ferric iron directly in the absence of reducing equivalents from NADPH, the CO-ferrous complex would be observed. However, this complex was not observed (data not shown), so it is unlikely that L-NHA provides

Table 1

Substrate	NO_2^- / NO_3^- (μM)		% Inhibition	% Average Inhibition
	air	CO / O_2 (80:20)		
L-Arginine	9.38	3.70	60.6	
	12.80	4.61	64.0	57.1 ± 6.5 (n=4)
	16.08	7.35	54.3	
	12.95	6.54	49.5	
L-NHA	17.86	12.54	29.8	
	14.83	10.84	26.9	
	14.71	10.46	28.9	32.3 ± 4.8 (n=6)
	20.81	12.63	39.3	
	18.58	11.77	36.7	
	17.60	11.94	32.2	

one electron to the ferric iron before oxygen binding. As a control, the addition of anaerobically prepared NADPH to the sample did produce the CO-ferrous complex as expected (data not shown). In addition, the addition of CO, PCD and PCA did not alter the absorbance spectrum of NOS (data not shown).

Discussion

The finding that NOS contained a P450 type heme suggests a reasonable mechanism for the conversion of L-arginine to L-NHA. Indeed, a role for the heme in catalysis was shown with L-arginine as the substrate as evidenced by CO inhibition studies (10, 16). Others have reached this same conclusion by CO studies although data was not given (11, 12). None of these studies, however, demonstrated the direct involvement of the heme in catalysis using L-NHA as a substrate, which is a question of significant mechanistic importance. The aim of this investigation was to determine if the heme was functioning in the oxidation of L-NHA to citrulline and NO. This question was examined through two approaches: by examining perturbations in the Soret band of NOS upon L-NHA binding as well as CO inhibition studies with L-NHA as a substrate.

Difference spectroscopy was used to investigate perturbations in the heme environment upon substrate binding (19). The addition of L-NHA to ferric NOS resulted in an increase at 384 nm and a decrease at 420 nm, which is characteristic of a type I spectral change. This is typically caused by a change in the spin state of the ferric iron of the heme from low-spin to high-spin. However, prior to substrate binding, the heme iron of NOS appears to be mostly in the high spin state with an absorbance maximum of 395-400 nm. Binding of L-NHA causes the absorbance maximum to shift to even shorter wavelengths, indicating a complete change to high spin. Therefore, L-NHA appears most likely to bind in proximity of the heme, as the spin state of the iron changes upon substrate binding.

The inhibition of P450 catalyzed reactions by CO has been mechanistically informative because it occurs via a tight complex formed by the reaction of ferrous iron with CO, although P450's show a varying susceptibility towards inhibition by CO (20-22). CO inhibited the NOS reaction with either L-arginine or L-NHA as substrates (Table I). However, the amount of inhibition afforded by CO with L-NHA was less than that observed with L-arginine (33% compared to 57%). The amount of inhibition observed with L-arginine in this study is less than that observed previously (10); however, the experimental conditions are different. The best comparison of NOS inhibition by CO would be to steriodogenic P450 enzymes, such as aromatase, lanosterol 14-demethylase, 17α-hydroxylase-C17,20-lyase and the cholesterol side-chain cleaving enzyme (P450$_{scc}$), since these enzymes are known to carry out multiple oxidative steps on substrates. However, the effect of CO on the individual steps in these reactions have not been studied extensively, and most reports use microsomes as the source of enzyme.

At least two possible explanations exist for the decreased CO sensitivity with L-NHA as a substrate. In the N-demethylation of ethylmorphine with liver microsomes, Estabrook proposed that the steady-state concentration of ferrous cytochrome P450, which was dependent on the rate of electron transfer from the reduced nucleotide to the ferric iron, was crucial in the sensitivity of a reaction towards CO (23). Although rates of heme reduction in the presence of L-arginine or

L-NHA are unknown, initial rate V_{max} data with both substrates are consistent with this idea since the V_{max} with L-NHA is approximately twice that of L-arginine (8, unpublished observations). The other explanation could simply be a difference in the K_d of O_2 with each substrate. If the K_d for O_2 with L-arginine is higher than that with L-NHA, CO could compete more easily with O_2 when L-arginine is the substrate and subsequently CO would inhibit to a higher degree with L-arginine versus L-NHA. In fact, results found with purified $P450_{scc}$ have shown successive decreases in the K_d's for O_2 in the presence of cholesterol, 22R-hydroxycholesterol and 20,22-dihydroxycholesterol (22).

Based on the existing evidence, a probable mechanism of NO formation from L-arginine involves two successive oxidations catalyzed by the heme. The first oxidation is a N-hydroxylation of L-arginine to produce L-NHA and the second is speculated to involve the formation of a ferric peroxide intermediate (18), which has been previously suggested to participate in aromatase (24) and lanosterol 14-demethylase (25) mechanisms. Nucleophilic attack on the guanido carbon would subsequently yield NO and citrulline (Figure 3). A ferric peroxide
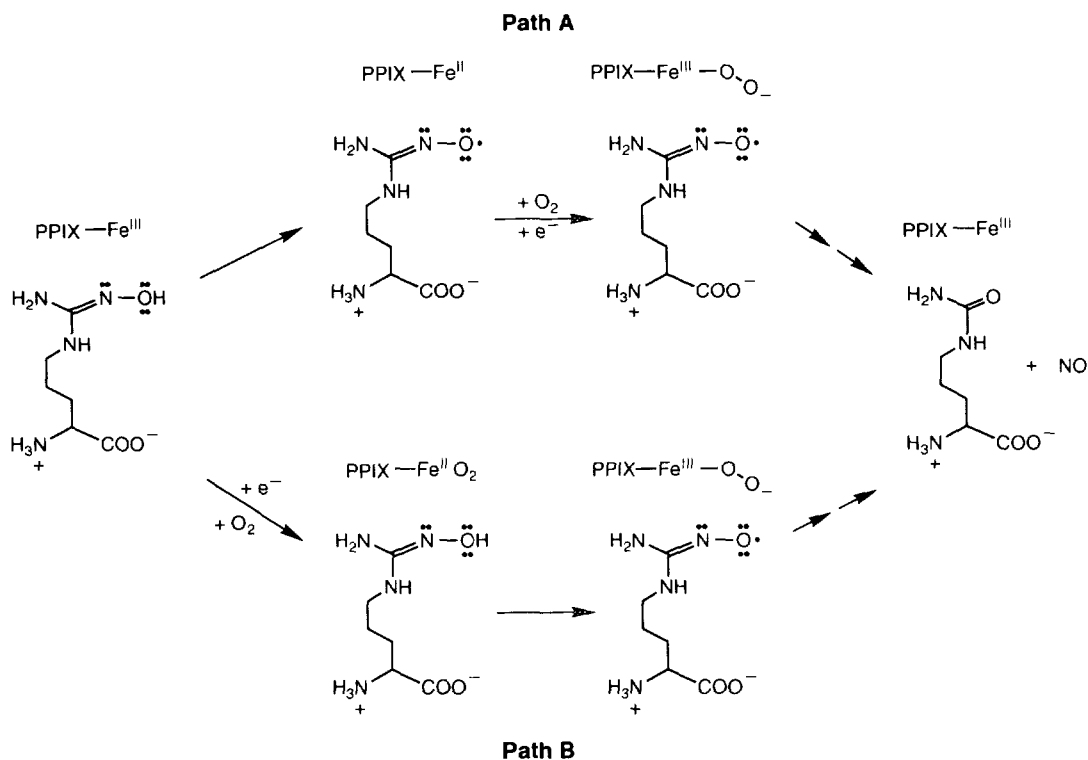

Figure 3. Two possible mechanisms for the oxidation of L-NHA to produce NO and citrulline involving the heme in catalysis are shown. In path A, the ferric iron is reduced to ferrous by one electron provided by the hydroxyguanidine moiety of L-NHA. Path B differs from path A in that the one electron provided by the oxidation of L-NHA occurs after the formation of the ferrous-dioxygen complex. Although not shown in this figure, in both paths A and B, the ferric peroxide could undergo nucleophilic attack on the guanido carbon to generate NO and citrulline. The mechanism depicted in path A would be expected to exhibit a ferrous-CO peak in the presence of anaerobic oxidized NOS, CO and L-NHA in the absence of NADPH. Protoporphyrin IX heme is represented as PPIX.

nucleophilic reaction has been observed with hepatic P450 (26) and chemical precedent for this type of reaction has also been reported (27). This mechanism also allows the requisite one-electron chemistry to occur which is necessary to produce NO. The formation of the ferric peroxide intermediate would be the result of one electron provided by the oxidation of L-NHA, from either the oxime oxygen or nitrogen and another from NADPH via the reductase domain. The one electron from L-NHA could reduce either the ferric iron (path A) or the ferrous-dioxygen complex (path B). The results reported here show that in the absence of NADPH, anaerobic NOS under a CO atmosphere did not form a ferrous-CO complex with the addition of anaerobic NHA. These results argue against the direct reduction of the ferric iron but instead suggest that the ferrous-dioxygen complex may be the species undergoing one electron reduction by L-NHA. Further experiments will be needed to prove whether this mechanism or still another is the actual mechanism of NO formation from the oxidation of L-NHA.

Acknowledgments

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