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Examination of *N*-hydroxylation as a Prerequisite Mechanism of Nitric Oxide Synthase Inactivation

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Abstract—L-N^5-(1-Hydroxyiminoethyl)-ornithine (L-NHIO) and L-N^6-(1-hydroxyiminoethyl)-lysine (L-NHIL) were synthesized and tested as potential intermediates in the mechanism-based inactivation of nitric oxide synthase (NOS) by L-N^5-iminoethyl-ornithine (L-NIO) and L-N^6-iminoethyllysine (L-NIL). Although these compounds were determined to be competitive inhibitors, mechanism-based inactivation was not observed. © 2000 Elsevier Science Ltd. All rights reserved.

Amino acid-based inhibitors of nitric oxide synthase (NOS) have shown promise in the treatment of many inflammatory diseases.[1–4] However, insufficient potency and selectivity for the inducible isoform (NOS II) limits the therapeutic potential of many NOS inhibitors.[5] Although binding selectivity to the NOS II isoform has been improved,[6–8] overall inhibitory selectivity is frequently confounded by mechanism-based inactivation.[9–12] Currently, very little is known about the fundamental mechanism for NOS II inactivation. The natural substrate, L-arginine, and the mechanism-based inactivator, L-N^G-methylarginine (L-NMMA), are similarly *N*-hydroxylated by NOS II to the intermediates L-N^G-hydroxyarginine and L-N^G-hydroxy-N^G-methylarginine, respectively.[13] Both L-N^G-hydroxyarginine and L-N^G-hydroxy-N^G-methylarginine display lower affinities for NOS II, but are metabolized more rapidly than their non-hydroxylated parent compounds.[13] Unlike L-N^G-hydroxyarginine, further metabolism of L-N^G-hydroxy-N^G-methylarginine results in mechanism-based inactivation.[13] This inactivation is associated with both covalent modification of the NOS protein and loss of NOS associated heme.[11,14] These effects on the NOS protein have been postulated to arise from the release of formaldehyde or nitrosomethane radical cation during the metabolism of L-N^G-hydroxy-N^G- methylarginine.[9,12–14] Unlike L-NMMA, inactivation by L-N^5iminoethyl-ornithine (L-NIO) and L-N^6-iminoethyl-lysine (L-NIL) is not associated with covalent modification of the NOS protein, but instead, is associated only with heme loss.[11,12] In addition, the release of formaldehyde or nitrosomethane radical cation that follows *N*-hydroxylation is not likely to occur in the case of L-NIO and L-NIL, because it would require a C–C instead of a C–N bond cleavage. Herein, we examined whether *N*-hydroxylation is a prerequisite for the inactivation of NOS by L-NIO and L-NIL.

Results and Discussion

Using eq 1 and PCNONLIN 4.2, we were able to describe the observed inhibition kinetics of L-NIO and L-NIL (Fig. 2A and B). The concentration dependence and saturability of enzyme inactivation, typical of mechanism-based inactivation, is apparent. Inactivation of NOS by L-NIO and L-NIL was dependent upon the presence of NADPH (data not shown). Based upon this analysis, the K_I/k_{inact} parameters of L-NIO and L-NIL were estimated to be 1.42 ± 0.34 μM/0.16 ± 0.01 min^{-1} and 2.16 ± 0.27 μM/0.35 ± 0.01 min^{-1}, respectively. These parameters are in close agreement with those previously reported for these compounds.[12] Unlike L-N^G-hydroxy-N^G-methylarginine, the presumed metabolic intermediates of NOS inactivation by L-NIO and L-NIL, L-NHIO

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and L-NHIL, did not inactivate NOS. Kinetic studies revealed that L-NHIO (Fig. 3A) and L-NHIL (Fig. 3B) are competitive antagonists of NOS. The K_I parameters of L-NHIO and L-NHIL were estimated to be 37.7 ± 8.12 μM and 41.1 ± 13.6 μM, respectively.

As previously reported for L-N^G-hydroxyarginine and L-N^G-hydroxy-N^G-methylarginine[20,21], these association constants are significantly higher than that observed for the non-hydroxylated counterparts (ANOVA $p < 0.05$). We conclude that, despite their ability to bind to the active site, L-NHIO and L-NHIL cannot inactivate the enzyme. These observations suggest that, unlike L-NMMA, *N*-hydroxylation is not a prerequisite mechanism of NOS inactivation for these analogues. This different interaction with the NOS enzyme coincides with the dissimilar mechanisms of inactivation reported for L-NMMA and these inhibitors.[9–12] This work is also consistent with the recent hypothesis that, in the presence of dioxygen, binding of L-NIO and L-NIL may, instead, induce the formation of reactive oxygen species that irreversibly modify the NOS protein.[12]

Experimental

Synthesis of (G), L-N^5-(1-hydroxyiminoethyl)-ornithine,[15] and (H), L-N^6-(1-hydroxyiminoethyl)-lysine[16] (Fig. 1). Compounds **G** and **H** were prepared from commercial starting materials **A** and **B** (Fig. 1). The protected ornithine and lysine derivatives **C** and **D** were reacted with ethyl *N*-hydroxyacetimidate[15] and the resulting hydroxyiminoethyl amino acids **E** and **F** were isolated by preparative TLC. After deprotection, using trifloroacetic acid (TFA), the final products (**G** and **H**) were purified by ion exchange chromatography[16] and recrystallized from aqueous ethanol.

L-N^5-(1-hydroxyiminoethyl)-ornithine (G). L-N^1-Boc-N^5-(1-hydroxyiminoethyl)-ornithine *tert*-butyl ester (**E**, 167 mg, 0.5 mmol) was mixed with 1.5 mL TFA. The reaction mixture was stirred in an ice bath for 2 h, then the TFA was removed in vacuo. The residue was dissolved in 3 mL water and the pH was adjusted to 7 with 2 N NaOH. This solution was applied to a Dowex 50W cation ion exchange column and the product was eluted with 200 mL of 1.5 M NH_4OH. The eluate was concentrated to 20 mL and adjusted to pH <2 with 2 N HCl. The solvent was removed in vacuo and the residue was crystallized from 90% ethanol to yield **G** (48 mg, 44.1%). ^{1}H NMR (D_2O): δ 1.45–1. (m, 2H), 1.68 (t, 2H), 1.91 (s, 3H), 3.19 (t, 2H), 3.5 (t, 1H). MS *m*/*z* 190 (MH^+). Anal. $C_7H_{15}N_3O_3$·HCl. Calcd: C, 37.26; H, 7.15; N, 18.62. Found: C, 37.12; H, 7.16; N, 18.38.

L-N^6-(1-hydroxyiminoethyl)-lysine (H). L-N^1-Boc-N^6-(1-hydroxyiminoethyl)-lysine *tert*-butyl ester (**F**, 180 mg, 0.5 mmol) was mixed with 1.0 mL TFA and 2 mL CH_2Cl_2. The reaction mixture was stirred in an ice bath for 2 h, and the product was isolated as described above for **G**. Recrystallization from aqueous ethanol yielded pure **H** (42 mg, 35.0%). ^{1}H NMR (D_2O): δ 1.45–1.50 (m, 2H), 1.65–1.70 (m, 2H), 1.91–1.94 (m, 2H), 2.10 (s, 3H), 3.36 (t, 2H), 3.78 (t, 1H). MS *m*/*z* 204 (MH^+). Anal. $C_8H_{17}N_3O_3HCl$. Calcd: C, 40.09; H, 7.57; N, 17.53. Found: C, 40.06; H, 7.60; N, 17.64.

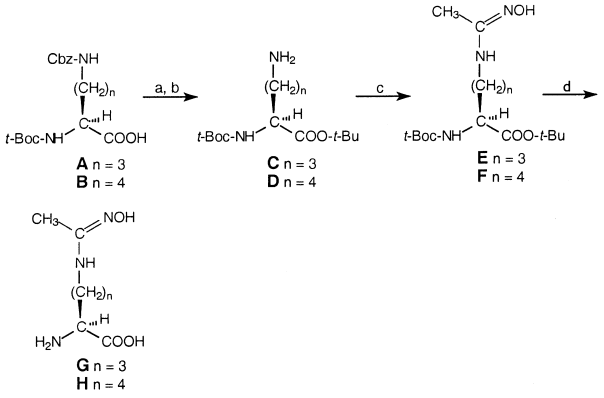

Figure 1. Synthesis of L-N^5-(1-hydroxyiminoethyl)-ornithine (**G**) and L-N^6-(1-hydroxyiminoethyl)-lysine (**H**). (a) H_2, Pd/C; (b) *t*-BuOH, EDCI, DMAP, CH_2Cl_2; (c) ethyl *N*-hydroxyacetimidate, 90 °C; (d) TFA.

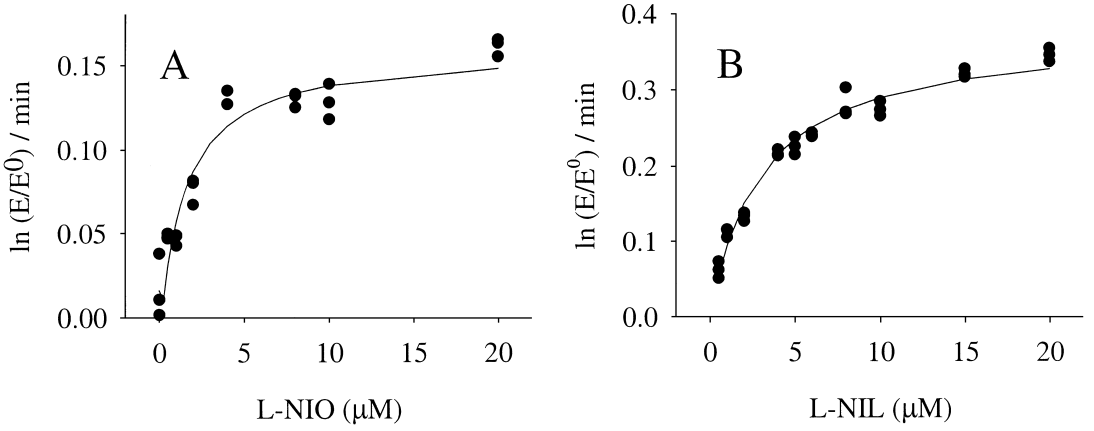

Figure 2. Inactivation kinetics of (A) L-N^5-iminoethylornithine and (B) L-N^6-iminoethyllysine for murine macrophage nitric oxide synthase. Symbols (●) represent the observed data of triplicate experiments. Line represents the fit obtained through these data using PCNONLIN 4.2 and eq 1.

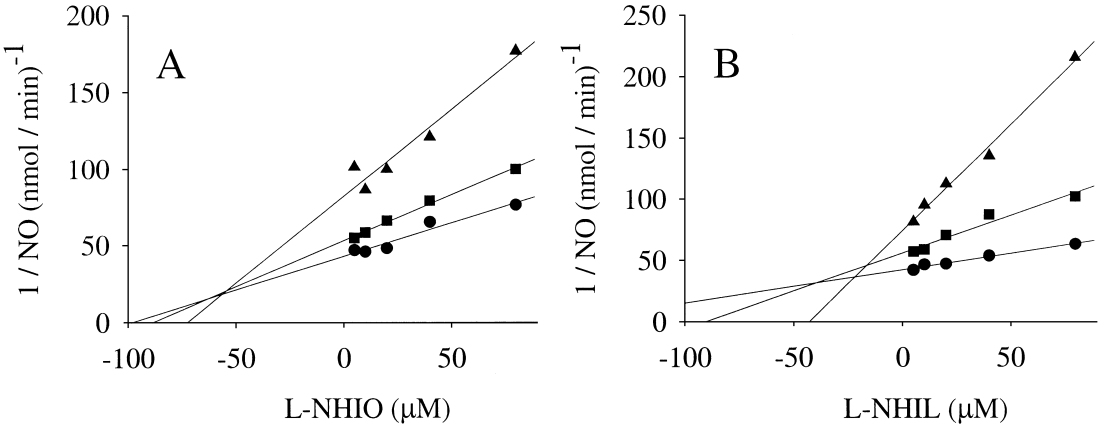

Figure 3. Dixon plots for the competitive antagonism of murine-macrophage nitric oxide synthase by (A) L-N^5-(1-hydroxyiminoethyl)-ornithine and (B) L-N^6-(1-hydroxyimino-ethyl)-lysine. Symbols represent the mean data of duplicate experiments obtained at Arg concentrations of (▲) 1.25, (■) 2.5, (●) 5.0 μM. Lines represent the best-fit linear regression through the observed data.

Mechanism-based inactivation studies

L-NIO, L-NIL, L-NHIO, and L-NHIL were evaluated for mechanism-based inactivation of purified murine macrophage nitric oxide synthase (NOS II, Cayman Chemical Co. Ann Arbor, MI). In a gas-tight reaction vial, NOS (0.1 units / 200 μL) was pre-incubated for 10 min at 37 °C with L-NIO, L-NIL (0, 1.25, 2.5, 5.0, 10, 15 and 20 μM), L-NHIO or L-NHIL (0, 1.25, 2.5, 5.0, 10, 15 and 20 and 30 μM) in 800 μL of 15 mM HEPES buffer, pH 7.4, containing 1 mM magnesium acetate, 0.1 mM NADPH, 60 μM tetrahydrobiopterin, 833 μM dithiothreitol. Following preincubation, 0.1 units of the enzyme (200 μL of the solution) was added to 800 μL of 15 mM HEPES buffer, pH 7.4, containing a final concentration of 2 mM L-arginine, 1 mM magnesium acetate, 0.15 mM NADPH, 24 μM tetrahydrobiopterin, 333 μM dithiothreitol, 100 units/mL superoxide dismutase (bovine erythrocyte). Following a 15-min incubation period, the amount of NO in the headspace of the reaction vial was quantified by chemiluminescence.[17] The inhibition constant (K_I) and the pseudo-first order inactivation rate constant (k_{inact}) were estimated using eq 1 and the nonlinear parameter estimation program PCNONLIN 4.2 (Statistical Consultants Inc. Lexington, KY). Equation 1 is a nonlinear form of an equation previously described by Kitz and Wilson.[18] The reported values of these parameters represent the mean ± S.D. of triplicate experiments.

$$\frac{\ln(E/E^0)}{t} = -\left(\frac{k_{inact}\cdot[I]}{[I]+K_I}\right) \quad (1)$$

E^0 represents the maximal enzyme activity following pre-incubation in the absence of inhibitor and E represents the maximal enzyme activity following pre-incubation with inhibitor for a certain time. [I] represents the concentration of the inhibitor to which the enzyme is exposed in the pre-incubation. NADPH dependence of inactivation by L-NIO and L-NIL (20 μM) was assessed by pre-incubation for 10 min at 37 °C in buffer which contained either 0.1 mM NADPH or $NADP^+$. At 0, 10, 20 and 30 min, the remaining enzyme activity was assessed by addition of 2 mM L-arginine, as described above.

Competitive inhibition studies

L-NHIO and L-NHIL were evaluated for competitive inhibition of NOS. In a gas-tight reaction vial, murine macrophage NOS (0.1 units) was incubated at 37 °C with L-NHIO or L-NHIL (5, 10, 20, 40 or 80 μM) and L-arginine (1.25, 2.5, or 5.0 μM) in 1 mL of 15 mM HEPES buffer, pH 7.4, containing 1 mM magnesium acetate, 0.1 mM NADPH, 12 μM tetrahydrobiopterin, 100 units/mL SOD (bovine erythrocyte) and 170 μM dithiothreitol. At 15 min, the headspace NO was determined by chemiluminescence[17]. Data were analyzed by the Dixon method.[19] The K_I value reported represents the mean ± SD of the value obtained by the three different substrate regression lines.

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