Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 528-534

N^{ω}-Hydroxy-L-Arginine: A Novel Arginine Analog Capable of Causing Vasorelaxation in Bovine Intrapulmonary Artery

Guillermo C. Wallace, Priya Gulati and Jon M. Fukuto*

Department of Pharmacology, UCLA School of Medicine
Center for the Health Sciences, Los Angeles, California 90024-1735

Received March 7, 1991

Summary: This study describes the effects of N^{ω}-hydroxy-L-arginine (NOHA) on endothelium dependent and endothelium independent relaxation of bovine pulmonary artery. These results are consistent with the hypothesis that NOHA is a biosynthetic intermediate in the production of nitric oxide from arginine. N^{ω}-Hydroxy-L-arginine causes both endothelium dependent and endothelium independent vasorelaxation, similar to that of arginine. This NOHA elicited relaxation was also inhibitable by N-methylarginine, N-nitroarginine and N-aminoarginine.

The physiological role of endogenously generated nitric oxide (NO) has been an area of intense recent interest. It has been shown to play a biological role in a variety of cells and tissues. For example, NO is an activator of guanylate cyclase and thus is involved in regulating vascular tone (1,2) and platelet aggregation and adhesion (3). NO is also generated by activated phagocytic cells and is involved in the cytotoxic action of these cells (4,5,6). Early studies on NO biosynthesis showed that NO is formed by the enzymatic oxidation of one of the terminal nitrogen atoms of the guanidinium function of arginine (5,7,8). The other product is citrulline. Molecular oxygen and NADPH are required for the oxidation along with, depending on the source of the enzyme, various other cofactors. Two mechanisms have been proposed for NO biosynthesis (5,9) and they are shown in **Figure 1**. Both mechanisms have in common an initial N-oxidation step to generate N^{ω}-hydroxy-L-arginine (NOHA). In order to determine the possible intermediacy of NOHA in the biosynthetic pathway of NO production, we have synthesized NOHA to test its biochemical and biological properties. We report here preliminary studies of the biological actions of NOHA on bovine artery preparations. If NOHA is an intermediate in NO biosynthesis, its actions should reflect those of the precursor, arginine.

*To whom correspondence should be addressed.

0006-291X/91 $1.50

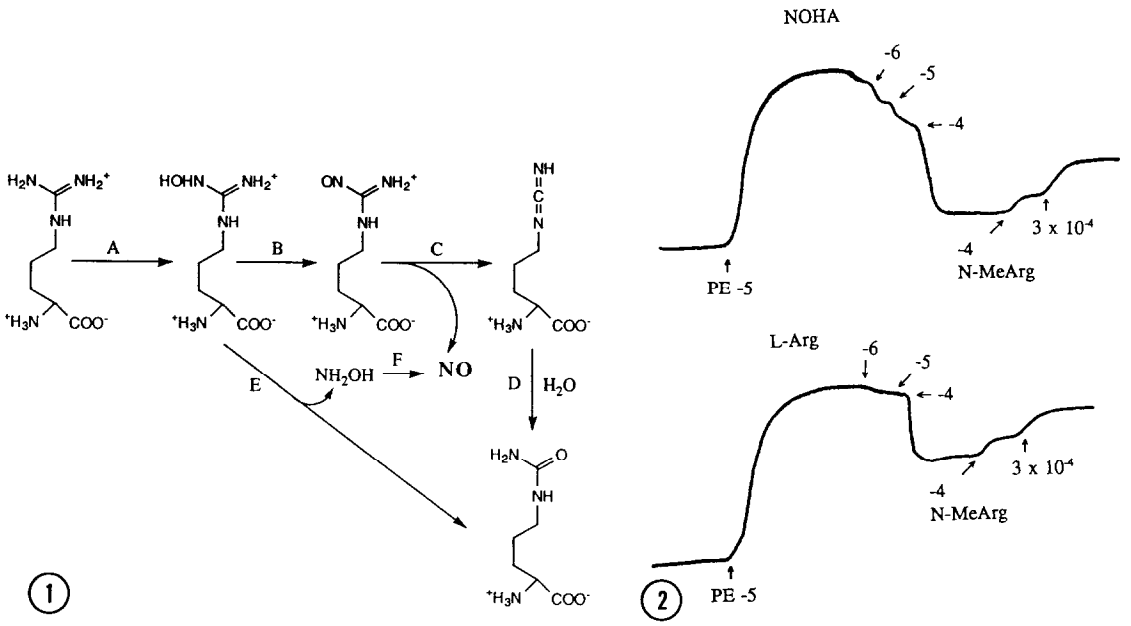

Fig. 1. Proposed pathways for the biosynthesis of nitric oxide from arginine.

Fig. 2. NOHA and L-Arginine dependent relaxation of intact, arginine depleted, bovine pulmonary artery. Isolated rings were precontracted with phenylephrine (PE) and challenged with NOHA and L-Arginine (L-Arg). Addition of N^{ω}-methyl-L-Arginine (N-MeArg) then followed. Concentrations are expressed as exponents to the base power of 10 and represent final bath concentrations.

Materials and Methods

Chemicals and solutions: L-Arginine, phenylephrine hydrochloride, acetylcholine, hemoglobin (human), methylene blue and N^{ω}-nitro-L-arginine were all purchased from Sigma (St. Louis, Mo.). N^{ω}-Hydroxy-L-arginine (NOHA) was synthesized according to the method to be described (10). Breifly, ornithine, protected on the α-amino (tBoc) and carboxylic acid (t-butyl ester), was reacted with cyanogen bromide in methanol. The resulting cyanamide was then reacted with hydroxylamine in dioxane to give diprotected N^{ω}-hydroxyarginine. Deprotection in trifluoroacetic acid resulted in NOHA. The compound was characterized by proton nuclear magnetic resonance spectroscopy, fast-atom bombardment mass spectroscopy, electron impact mass spectroscopy and exact mass measurement (10). N^{ω}-methyl-L-arginine was synthesized according to the general method of Patthy et al. (11). N^{ω}-Amino-L-arginine was synthesized according to the method described earlier (12). Hemoglobin was prereduced with dithionite before use. Krebs-bicarbonate solution consisted of 118 mM NaCl, 1.5 mM $CaCl_2$, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11 mM glucose and 0.023 mM EDTA.

Arterial smooth muscle relaxation: Bovine intrapulmonary rings were prepared and changes in isometric force measured as described previously (13). All tissues were submaximally precontracted using phenyephrine before being challanged by the substrates. In the experiments requiring endogenous arginine depletion, tissues were suspended under 4 grams of tension in oxygenated (95% O_2/5% CO_2) Krebs solution for 12-14 hours. Arginine depletion was confirmed by the lack of acetylcholine response. In endothelium denuded preparations, the endothelial cells were removed by the method described previously (14). Endothelial cell loss from the denuded tissues was confirmed by the lack of response to acetylcholine in freshly mounted tissues. Test compounds were added directly to the tissue baths and all reported

concentrations refer to the final concentrations in the baths. Inhibitors were added to the baths after the response to NOHA or arginine had been determined. All experimental protocols were repeated, in triplicate, at least 3 times using fresh tissue. Figures in the text represent a typical experiment.

Results

The effect of NOHA on bovine intrapulmonary artery preparations were compared directly to the actions of arginine under the following experimental protocols.

Arginine depleted intact arterial rings (day 2): Both NOHA and arginine were capable of eliciting significant vasorelaxation in a concentration dependent manner in submaximally precontracted arginine depleted tissues (**Figure 2**). The relaxing effect of NOHA in the depleted tissues was much greater than the effect in freshly mounted (day 1) tissues. NOHA was a slightly more potent vasorelaxing agent than arginine. In both cases, the relaxation was reversible with the addition of either N-nitro, N-amino or N-methylarginine in a concentration dependent manner.

Endothelium denuded, arginine depleted (day 2) tissues: In endothelium denuded, arginine depleted tissue, both NOHA and arginine were capable of eliciting vasorelaxation in a concentration dependent manner (**Figure 3**). Both NOHA and arginine induced relaxation

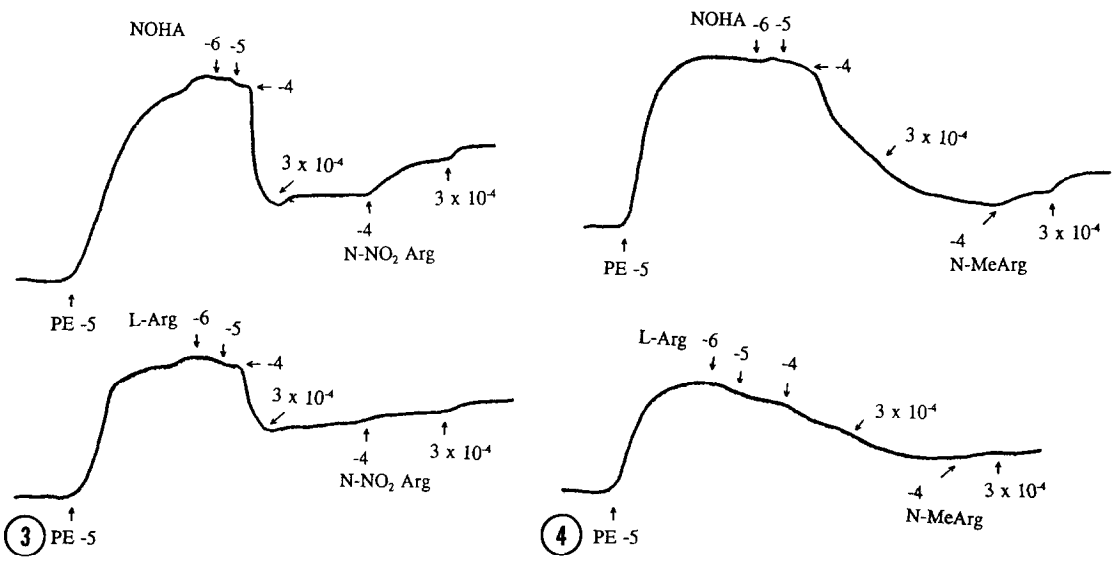

Fig. 3. NOHA and L-Arginine dependent relaxation of endothelium denuded arginine deplete bovine pulmonary artery. Isolated rings were precontracted with phenylephrine (PE) and challenged with NOHA and L-Arginine (L-Arg). Addition of N^GNitro-L-Arginine (N-NO_2 Arg) then followed. Concentrations are expressed as exponents to the base power of 10 and represent final bath concentrations.

Fig. 4. NOHA and L-Arginine dependent relaxation of intact, fresh bovine pulmonary artery. Isolated rings were precontracted with phenylephrine (PE) and challenged with NOHA and L-Arginine (L-Arg). Addition of N^G-methy-L-Arginine (N-MeArg) then followed. Concentrations are expressed as the exponents to the base power of 10 and represent final bath concentrations.

were inhibitable by N-nitro, N-amino and N-methylarginine. NOHA was consistently more potent in causing vasorelaxation compared to arginine.

Intact arterial rings in fresh tissue (day 1): Both NOHA and arginine were tested for their ability to relax tissue (not depleted of arginine). NOHA was capable of causing slight, yet discernable, vasorelaxation in a concentration dependent manner in freshly mounted, submaximally precontracted tissue whereas exogenously added arginine caused little or no vasorelaxation (**Figure 4**). The relaxation response caused by NOHA was reversed by the addition of either N-nitro, N-amino or N-methylarginine.

Inhibition by methylene blue and oxyhemoglobin in arginine depleted tissues (day 2): NOHA addition (1×10^{-4} M) caused the expected vasorelaxation in arginine depleted tissues. The tissues were then washed three times with Krebs solution and again submaximally precontracted with phenylephrine. Hemoglobin, prereduced with dithionite, was then added to the bath at 1×10^{-6} M. Addition of NOHA at concentrations as high as 3×10^{-4} M caused no vasorelaxation (data not shown). The identical procedure was used for methylene blue (1×10^{-5} M) and it also completely blocked the effects of NOHA at concentration as high as 3×10^{-4} M (data not shown).

Discussion

The mechanistic intricacies of the enzymatic conversion of arginine to citrulline and NO have yet to be elucidated. All that is currently known about the biosynthetic pathway is that NO originates from an oxygen and NADPH dependent oxidation of a terminal guanidinium nitrogen on arginine. In spite of the limited information regarding NO biosynthesis, two mechanisms have been postulated (**Figure 1**). One pathway (A-B-C-D, **Figure 1**) proposed by Marletta et al. (5) involves an initial oxidation of arginine to give NOHA which then undergoes another oxidation to generate the corresponding nitroso compound. This compound then undergoes C-N bond cleavage, releasing NO. The other mechanism (A-E-F, **Figure 1**), proposed by DeMaster et al. (9), also involves an initial N-hydroxylation to give NOHA which is then hydrolyzed to generate citrulline and hydroxylamine. Hydroxylamine is then oxidized by another enzyme (possibly catalase) to give nitric oxide. It has become clear, at least in the macrophage, that neither mechanism is entirely correct since Kwon and coworkers have demonstrated that the oxygen atom which ends up in citrulline comes from dioxygen and not water (15). However, both pathways propose, as their first step, the monohydroxylation of a terminal guanidinium nitrogen on arginine to generate NOHA. If NOHA were a biosynthetic intermediate, it should cause vasodilation in all instances arginine does (assuming that NOHA, when added within the cell). It is also possible that the vasorelaxing properties of NOHA will exceed those

of arginine itself if the rate determining process for NO formation from arginine precedes NOHA formation.

It has been determined that in *in vitro*, tissue response assays, exogenously added arginine has little or no biological effect on freshly mounted tissues. Presumably, this is due to the fact that normal arginine concentrations in the endothelial cells are well above the V_{max} concentration of the enzyme or enzymes responsible for NO synthesis from arginine. However, when the cells are depleted of arginine, addition does cause significant vasodilation (16). The results presented herein (**Figure 2**) indicate that NOHA is also capable of causing vasodilation in arginine depleted bovine pulmonary artery (with endothelium) and that the potency of NOHA is greater than that of arginine itself. NOHA appears to be acting by a nitric oxide dependent pathway since NOHA dependent relaxation is abolished by oxyhemoglobin and methylene blue and the NOHA dependent relaxations were inhibited by several of the known NO synthesis inhibitors (N-methyl, N-nitro and N-aminoarginine). These inhibitors have all been shown to inhibit arginine-dependent relaxations as measured by tissue response, decreased cGMP levels and decreased NO levels (measured as nitrite) (12,17-19).

It has been shown that smooth muscle cells, as well as endothelial cells, are capable of producing NO, by an arginine dependent process (20). We, therefore, investigated the effect of NOHA on endothelium denuded, arginine depleted bovine intrapulmonary artery. NOHA, like arginine, is capable of causing relaxation in these denuded tissue preparations in a concentration dependent manner which is inhibitable by N-methyl, N-nitro and N-aminoarginine (**Figure 3**). The potency of NOHA in causing relaxation in these tissues also was consistently greater than that of arginine.

In spite of the inherent variability of the *in vitro* assays, NOHA was consistently more potent than arginine in causing vasorelaxation in both the previous experimental protocols (arginine depleted tissue with and without endothelium). Therefore, the effect of NOHA was tested on freshly mounted, intact tissues which were not depleted of arginine. NOHA was capable of eliciting slight vasorelaxation even in fresh tissues whereas arginine had little or no effect (**Figure 4**). Thus, if NOHA is a metabolic intermediate in NO biosynthesis, it is possible that its formation is either rate determining or occurs after the rate determining step.

Although the results of this study are consistent with NOHA serving as a metabolic intermediate, there are other possible explanations which have yet to be ruled out. For example, it is possible that NOHA produces NO by a completely independent mechanism and that the inhibitors (N-methyl, N-nitro and N-aminoarginine) fortuitously inhibit that process as well. Alternatively, NOHA might simply be an alternate substrate for the enzyme or enzymes responsible for NO generation. Although, further studies are required to establish NOHA as an endogenous metabolic intermediate NOHA is shown here to be an arginine related

compound capable of eliciting vasorelaxation with greater potency than the endogenous amino acid substrate, arginine.

The effect of the inhibitors on NOHA dependent relaxation is noteworthy. If NO biosynthesis from arginine does involve initial monohydroxylation of a terminal guanidinium nitrogen to give NOHA, as the proposed mechanisms suggest (**Figure 1**), then the inhibitors cannot be acting at this initial step. The fact that N-methyl, N-nitro and N-aminoarginine inhibit both NOHA dependent relaxation and arginine dependent relaxation means these inhibitors are likely to be acting at a step in the biosynthetic pathway subsequent to NOHA formation. Alternatively, arginine is oxidized to NOHA by the protein and NOHA is not released. It is then further oxidized in the active site. Thus, inhibitors of arginine binding will also block NOHA binding to the same protein.

Acknowledgments

The authors would like to thank Russell Byrns and Georgette Buga for their expert technical assistance and especially thank Dr. Louis J. Ignarro for his helpful discussions throughout the course of this work.

References

1. Palmer, R. M. J., Ferrige, A. G. and Moncada, S. (1987) Nature (London), 288, 373-376.
2. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G. (1987) Proc. Natl. Acad. Sci. USA, 84, 9265-9269.
3. Radomski, M. W., Palmer, R. M. J. and Moncada, S. (1987) Br. J. Pharmacol., 92, 181-187.
4. Hibbs, J. B., Taintor, R. R., Vavrin, Z. and Rachlin, E. M. (1988) Biochem. Biophys. Res. Commun., 157, 87-94.
5. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. and Wishnok, J. S. (1988) Biochemistry, 27, 8706-8711.
6. Stuehr, D. J., Kwon, N. J., Gross, S. S., Thiel, B. A, Levi, R. and Nathan, C. F.,(1989) Biochem. Biophys. Res. Commun., 161, 420-426.
7. Iyengar, R., Stuehr, D. and Marletta, M. A. (1987) Proc. Natl. Acad. Sci. USA, 84, 6369-6373.
8. Palmer, R. M. J., Ashton, D. S. and Moncada, S. (1988) Nature (London), 33, 664-666.
9. DeMaster, E. G., Raij, L., Archer, S. L. and Weir, E. K. (1989) Biochem. Biophys. Res. Commun., 163, 527-533.
10. Wallace, G. C. and Fukuto, J. M. (1991) in preparation.
11. Patthy, A., Bajusz, S. and Patthy, L. (1977) Acta Biocheim. et Biophys. Acad. Sci. Hung., 12, 191-196.
12. Fukuto, J. M., Wood, K. S., Byrns, R. E. and Ignarro, L. J. (1990) Biochem. Biophys. Res. Commun., 168, 458-465.
13. Ignarro, L. J., Byrns, R. E. and Wood, K. S. (1987) Circ. Res., 60, 82-92.

14. Gold, M. E., Buga, G. M., Wood, K. S., Byrns, R. E., Chaudhuri, G. and Ignarro, L. J. (1990) Circ. Res., 66, 355-366.
15. Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E. and Stuehr, D. J. (1990) J. Biol. Chem., 265, 23, 13442-13445.
16. Gold, M. E., Bush, P. A. and Ignarro, L. J. (1989) Biochem. Biophys. Res. Commun., 164, 714-721.
17. Johns, R. A., Peach, M. J., Linden, J. and Tichotsky, A. (1990) Circ. Res., 67, 979-985.
18. Rees, D. D., Palmer, R. M. J., Schulz, R., Hodson, H. F. and Moncada, S. (1990) Br. J. Pharmacol., 101, 746-752.
19. Ishii, K., Kerwin, J. F. and Murad, F. (1990) Can. J. Pharmacol., 68, 749-751.
20. Wood, K. S., Buga, G. M., Byrns, R. E. and Ignarro, L. J. (1990) Biochem. Biophys. Res. Commun., 170, 80-88.