N^{ω}-HYDROXY-L-ARGININE AND ITS HOMOLOGUES. CHEMICAL AND BIOLOGICAL PROPERTIES. A REVIEW

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

N^{ω}-Hydroxy-L-arginine (NOHA) is a stable intermediate in NO formation from L-arginine catalyzed by NO synthase (NOS). Apparently, NOHA can be released and serve as a stable reserve NO donor (as a substrate of NOS) or transported and exert its own biological effects. It shows endothelium-dependent as well as endothelium-independent vasorelaxant activity. The latter case indicates that NOHA can be metabolized by pathways independent of NOS. These possibilities are discussed in detail. Of the available NOHA homologues homo-NOHA is a good substrate of NOS while nor-NOHA seems to be a very poor substrate of this enzyme. On the contrary, nor-NOHA exerts arginase inhibitory activity 20 times higher than NOHA whereas homo-NOHA is inactive. Detailed investigation of biological activities of NOHA and its homologues seems to be promising from the pharmacological point of view. A review with 43 references.

Keywords: N^{ω}-Hydroxy-L-arginine; N^{ω}-Hydroxy-L-arginine homologues; Nitric oxides; Drug research; Cardiovascular research; Amino acids; NO synthase.

1. INTRODUCTION

With the exception of capillaries, the wall of blood vessels consists of three layers: tunica intima, tunica media and tunica adventitia. Their dysfunction can manifest itself as an impairment in the production of nitric oxide (NO), a molecule that is known to mediate vasorelaxation[1]. This occurs in various pathophysiological situations (endothelial dysfunction is implicated in atherosclerosis, hypertension or diabetes and adventitia-derived NO contributes to hyperactivity in vessels exposed to pro-inflammatory stimuli). A selective delivery of NO to the individual vascular layers, using appropriate compounds, may thus represent an interesting and promising tool in the cardiovascular research by avoiding negative effects of NO, such as excessive vasodilation, hypertension or production of cytotoxic molecules.

Indeed, oxidative cleavage of various compounds that contain a $C(NH_2)$=NOH function, resulting in generation of stable nitrogen oxides (NO_x), with the possible intermediate formation of NO, has been described[2,3]. These compounds include *N*ω-hydroxy-L-arginine (NOHA). NOHA is an intermediate in biosynthesis of NO from L-arginine, catalyzed by NO synthase (NOS). Several alternative pathways for its oxidation have been proposed which include the involvement of cytochrome P-450 or other hemoproteins and of superoxide[4–6]. The existence of such a pathway might be important in blood vessels to restore NO formation in various pathological situations in which endothelial NOS expression or activity is impaired.

2. NOHA

It is now well established that NOHA is an intermediate in biosynthesis of NO from L-arginine, catalyzed by NO synthase[7]. The NO/L-arginine pathway is resumed in Fig. 1. For unknown reasons, NOHA can be liberated

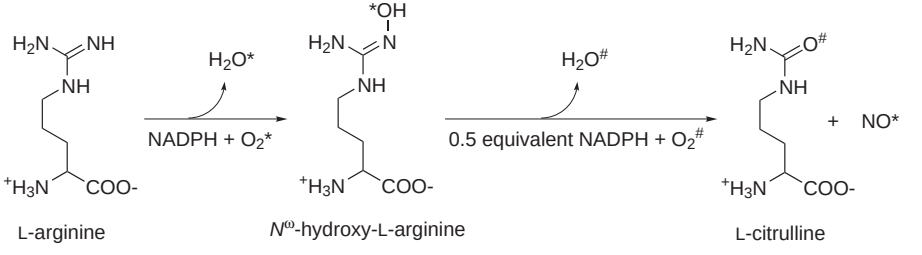


FIG. 1
L-Arginine/NO pathway catalyzed by NO synthase (* and # determine the origin of oxygen)

from the active site of NOS. Besides, it has been suggested that it can circulate and exert paracrine effects (i.e. effects exhibited in other cells different from those in which produced)[8]. As it can be used as substrate by NOS, it has been suggested that it can serve as a stable NO donor[9]. Cells with elevated NOS-II expression, such as EMT-6 mammary adenocarcinoma cells stimulated with bacterial lipopolysaccharide/interferon-γ, release substantial amounts of NOHA into the extracellular space[8]. Additionally, in lipopolysaccharide-treated rats, a significant elevation in serum concentration of NOHA has been reported[10], suggesting that circulating NOHA might represent a specific marker of NOS activity in vivo. In humans, NOHA concentration has been shown to be important in pathophysiology. Indeed, in inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus, serum NOHA was significantly increased, suggesting an upregulation of NOS activity or expression[11]. In contrast, NOHA is reduced in the plasma of patients with a metabolic syndrome who represent a group with extensive cardiovascular risk factors for the development of atherosclerosis[12].

2.1. Chemical Reactivity of NOHA

NOHA is subject to decomposition under either basic or oxidizing conditions. In fact, one of the dominant chemical features of NOHA is its ability to act as a reducing agent. Its *N*-hydroxyguanidine function is electron-rich and can be easily oxidized. Oxidation of NOHA can lead either to the release of HNO (from a two-electron oxidation) or to the formation of an apparent radical species $NO^{\bullet}$ (from one-electron oxidation)[13]. Unless otherwise indicated, the term nitric oxide (NO) will be used here to design either of the nitrogen monoxides ($NO^{\bullet}$, NO^{+}, NO^{-}).

The possibility of superoxide-induced oxidative cleavage of the C=NOH bond of NOHA has been suggested by Sennequier[14] who based his hypothesis on the similarity with the cleavage of the C=NOH bond of *N*-hydroxy-(aryl)guanidines upon reaction with KO_2. Similarly, in aqueous solution, NOHA is converted to nitrite and nitrate by superoxide with NO as a possible intermediate[6].

Using the xanthine/xanthine oxidase system for the production of superoxide anion, Modolell et al.[15] have also demonstrated the ability of superoxide to cleave NOHA to nitrite. Additionally, as demonstrated in the same report, the superoxide produced during the induction of a respiratory burst in several types of cells (murine bone marrow-derived macrophages from NOS-II knock out and wild-type mice) was capable of producing nitrite by

the cleavage of NOHA. Both in the xanthine/xanthine oxidase system and macrophages, the nitrite production was completely abolished by addition of SOD indicating that superoxide is the reactive agent. Based on the measurement of nitrite, the authors proposed the existence of a superoxide-mediated conversion of NOHA to NO.

However, the probability of the reaction between superoxide and NOHA under physiologically important conditions (superoxide produced during NOS and P-450 catalytic activity, for instance) seems to be low for two reasons: (i) at the physiological pH of 7.4, only 21% of NOHA exists in the deprotonated $C{=}NO^-$ form, and therefore only a fraction of NOHA would be amenable to the oxidative cleavage by superoxide and (ii) the rate of the reaction (O_2^- + $C{=}NO^-$) is too slow to compete with reactions such as the one between superoxide and the NO radical or SOD-catalyzed dismutation of superoxide. Additionally, the reaction between NOHA and superoxide ion does not produce L-citrulline (besides NO) but a cyclic carbodiimide, suggesting that this pathway may be more important for other compounds[16]. However, these findings were not confirmed by results of Chalupský et al.[17] who found no cyclic carbodiimide but L-citrulline as the main product of the reaction.

2.2. Enzymic Pathways of NOHA

The ability of NOHA to act as a good substrate of NOS has been demonstrated using the purified recombinant neuronal and macrophage isoforms[18] and the endothelial isoform[19]. Additionally, when using the macrophage NOS, the apparent K_m observed with NOHA is similar to the value observed with L-arginine (6.6 and 2.3 μmol/l, respectively)[7].

Furthermore, several NOS-independent enzymic pathways of NOHA have been reported in the literature, as described below. The oxidation of NOHA to NO by hemoproteins might be important for the formation of NO in cells or compartments not containing NOS, suggesting that NOHA would thus act as a transportable precursor of NO [20].

Liver microsomes from rats treated with dexamethasone, an inductor of the cytochrome P-450 3A subfamily, exhibited a particular activity towards NOHA as shown by L-citrulline and nitrogen oxides production and thus, the cytochrome P-450 3A subfamily has been proposed to catalyze the oxidative cleavage of NOHA [4]. In addition, SOD inhibited the P-450-dependent oxidation of NOHA to a great extent, suggesting that the reaction of P-450-generated superoxide with NOHA would lead to L-citrulline and NO in these microsomes. NOHA is not a high-affinity substrate for this P-450

since it is not as well positioned in the active site of P-450 3A as in the NOS active site[5]. However, other authors reported the occurrence of oxidation by rat liver microsomes of compounds involving a C=N(OH) function (including *N*-hydroxyguanidines, amidoximes, ketoximes and aldoximes) with the release of nitrogen compounds, such as NO, NO_2^- and NO_3^-. A general mechanism of such oxidative clevages of C=N(OH) bonds by cytochromes P-450 and NOS, with the involvement of superoxide anion and its Fe(III) complex [(FeIII-O_2^-) or (FeII-O_2)] as main active species has been proposed[3]. Whether this mechanism can account for generation of sufficient amounts of NO to produce physiological effect in vivo remains to be elucidated.

In cultured rat aortic smooth muscle cells, NOHA induced a dose-dependent nitrite production that was not inhibited by a NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). However, this nitrite production was abolished by miconazole, an inhibitor of cytochrome P-450[21], supporting the hypothesis that vascular smooth muscle cell cytochrome P-450 might produce NO from NOHA. Additionally, expression of cytochrome P-450 1A and 1B was detected in vascular smooth muscle cells of mice[22].

Further, Boucher et al.[20] demonstrated that horse radish peroxidase catalyzes the oxidation of NOHA with H_2O_2 under the formation of L-citrulline and nitrite. The L-citrulline and nitrite production was inhibited by azide and cyanide, inhibitors of horse radish peroxidase.

2.3. NOHA:NO Adduct

The first report of a reaction between NOHA and NO has been brought in 1991. Using a bioassay cascade system, Zembowicz et al.[23] showed that cultured endothelial cells produce a vasodilator substance from NOHA which is distinct from NO and that NOHA reacts chemically with authentic NO or endothelial cells-derived NO to form a potent and more stable vasodilator. The hypothesis involving a reaction of NOHA with NO was supported by the following findings: (i) HPLC analysis of the reaction products of NOHA and NO revealed the quantitative formation of a labile compound, (ii) the corresponding HPLC fraction had vasorelaxant properties, (iii) the biological activity of the substance was inhibited by oxyhemoglobin, and (iv) it readily released NO in water at elevated temperatures.

A similar result has been reported upon reaction of hydroxyguanidine and NO. When bubbling NO through the solution of hydroxyguanidine, a new compound was formed. It had similar physico-chemical properties to those of the previously described NOHA:NO adduct. It was also a potent

vasodilator and its action was inhibited by oxyhemoglobin, suggesting that the hydroxyguanidine moiety of NOHA is essential for the reaction with NO [24].

Furthermore, a stable relaxing substance that is not identical to NO is released from cytotoxic-activated macrophages. It seems to be synthesized from L-arginine and acts through the activation of soluble guanylyl cyclase[25].

In a cascade superfusion bioassay system, the effluate from cytokine-stimulated vascular smooth muscle cells induced a stable relaxation. The properties of the effluate were compared with those of the NOHA:NO adduct that was instantaneously generated upon reaction of NOHA with authentic NO. The pharmacological profile of this synthetic adduct was indistinguishable from that of the vascular smooth muscle cells effluate, as judged by comparative bioassay with different vascular and nonvascular smooth muscle cells preparations. Two other biologically relevant NO derivatives, *S*-nitrosothiols or dinitrosyliron complexes, were not detectable in the effluate[26].

2.4. Vasorelaxant Effects of NOHA

The first study dealing with vasorelaxing properties of NOHA appeared in 1991. Wallace et al.[27] described the NOHA-induced relaxation of bovine pulmonary artery under three conditions: (i) intact, arginine-depleted arterial rings, (ii) endothelium-denuded, arginine-depleted tissues and (iii) intact, fresh tissues. In all these preparations, NOHA was capable of causing a concentration-dependent vasorelaxation that was reversed by addition of several of the known NO synthesis inhibitors (N^{ω}-nitro, N^{ω}-amino or N^{ω}-methylarginine), supporting the hypothesis that NOHA is a substrate of NOS. However, it has to be noted that the endothelium-independent relaxation produced by NOHA was also produced by L-arginine. Furthermore, both were observed after prolonged incubation of the vessels in the organ baths, suggesting the likely induction of NOS-II. Unfortunately, the possible occurrence of NOS-II activity has not been studied in these experiments, as the NOS-II induction in blood vessels was not documented at the time.

The endothelium-dependent relaxation induced by NOHA was further investigated by Zembowicz et al.[28]. Based on experiments using a bioassay cascade system, three mechanisms by which NOHA induces endothelium-dependent relaxations were proposed (Fig. 2): (i) NOHA is a substrate for the constitutive NOS present in endothelial cells, (ii) it reacts with NO released from endothelial cells to form a potent and more stable vasodilator,

and (iii) it induces a relatively stable, endothelium-dependent relaxation that is not blocked by the inhibitors of NO synthesis. In these experiments, infusions of NOHA over the bioassay tissues (endothelium-denuded rabbit aortic rings) had no direct effect on their tone[23].

Similar cascade experiments were performed by Swierkosz et al.[29] who demonstrated that under flow conditions (NOHA perfusing a column of endothelial cells), NOHA forms a substance which relaxes rabbit aortic strips. The substance released from the endothelial cells is NO or a closely related molecule and is formed by a pathway which depends on a constitutive, calcium-dependent enzyme and is not inhibited by L-NAME.

As reported by Gibson et al.[30], NOHA had no direct effect on muscle tone of the mouse anococcygeus. In contrast, no evidence was found for an interaction between NOHA and NO in the tissue, since NOHA had no effect on relaxations by exogenous NO.

In isolated porcine large coronary artery, NOHA evoked strong, endothelium-dependent dilation which was eliminated by L-NAME but not affected by cytochrome P-450 inhibitors (miconazole or 7-ethoxyresorufin), suggesting that NOHA affects the contractility of coronary arteries by acting as a substrate for the endothelial NOS but has no direct effect on the smooth muscle cells in this tissue[31].

Endothelium-independent relaxation of rat aorta induced by NOHA was also studied by Vetrovsky et al.[32]. Activation of guanylyl cyclase and NO formation was implicated in this relaxation. Apparently 7-ethoxyresorufin-sensitive NAD(P)H-dependent pathway was involved.

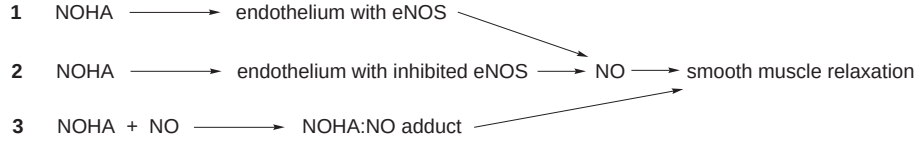


FIG. 2
Mechanisms of the endothelium-(in)dependent relaxation induced by NOHA[28]. 1 NOHA is a substrate for the constitutive NOS present in endothelial cells. 2 NOHA can be converted to NO by NOS-independent pathway(s). 3 NOHA can form with NO a more stable vasorelaxant, NOHA:NO adduct

3. THE HOMOLOGUES OF NOHA

3.1. Structure of NOHA Homologues

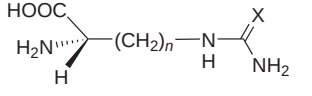
The structure of NOHA homologues is presented in Fig. 3. As shown in the figure, N^{ω}-hydroxynor-L-arginine (nor-NOHA) is a shorter analogue of NOHA while lengthening of the NOHA chain by one CH_2 leads to N^{ω}-hydroxyhomo-L-arginine (homo-NOHA).

3.2. Substrate Specificity of NOS with Respect to NOHA Homologues

As reported by Moali et al.[18], L-arginine and NOHA led to high amounts of nitrite in the presence of purified recombinant neuronal and macrophage NOS. Their longer homologues, homoarginine and homo-NOHA, also appeared to be good substrates for both NOS isoforms. However, quite interestingly, nor-NOHA seemed to be a very poor substrate for these NOS, as it did not produce significant amounts of nitrite. Available enzyme kinetic data of L-arginine, NOHA and their homologues are summarized in Table I.

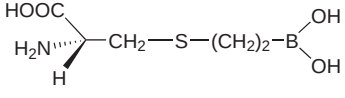
4. INHIBITION OF ARGINASE BY NOHA AND ITS HOMOLOGUES

Arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea. At least two distinct and nonhomologous arginase genes exist in mammals, and their products are termed arginase-I and arginase-II for the hepatic and extrahepatic isoforms, respectively[36]. Because extrahepatic tissues do not possess a complete urea cycle, the function of arginase-II in such tissues is

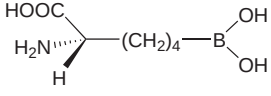


X = NH
X = N–OH

n = 4: homoarginine
n = 3: arginine
n = 2: norarginine
n = 4: homo-NOHA
n = 3: NOHA
n = 2: nor-NOHA



S-(2-boronoethyl)-L-cystein (BEC)



(2*S*)-amino-6-boronohexanoic acid (ABH)

FIG. 3
Structures of L-arginine homologues, their hydroxy derivatives and structures of arginase inhibitors

unclear. Interestingly, the hepatic arginase-I has been shown to be constitutively expressed in the endothelium and smooth muscle of coronary arterioles[37]. Furhermore, an increase in arginase activity accompanied by a selective increase in arginase-I mRNA was induced by mechanical forces in aortic smooth muscle cells[38].

NOHA is a potent inhibitor of the hydrolysis of L-arginine to L-ornithine catalyzed by purified rat liver arginase[39]. Nor-NOHA has been reported to be about twenty-fold more potent than NOHA to inhibit arginase in both unstimulated and (IFN-γ + lipopolysaccharide)-activated murine macrophages[40]. A comparison of some of the known arginase inhibitors is presented in Table II.

As both NOS and arginase compete for the same substrate, the possibility of reciprocal regulation of both arginine metabolic pathways has been recently explored. Substantial constitutive arginase activity has been found in rat aortic endothelial cells and a different isoform of arginase was induced by lipopolysaccharide. The intracellular arginase activity can be markedly inhibited because of NOHA formation. This inhibition may be a mechanism ensuring sufficient arginine availability for high-output production of NO [43]. Another example of a reciprocal regulation of both arginine pathways has been brought by Kim et al.[42]. The functional enhancement of neurogenic relaxation in penile cavernosal tissue exposed to the potent arginase

TABLE I
Enzyme kinetic data of L-arginine and *N*-hydroxy-L-arginine and their homologues (oxidation by iNOS)[33–35]

Compound	iNOS		
	K_m, μmol/l	k_{cat}, 1/min	k_{cat}/K_m 1/(min × μmol/l)
L-Arginine	5.9	84.6	14.3
Homo-L-arginine	29	[a]	[a]
Nor-L-arginine	[b]	[b]	[b]
N-Hydroxy-L-arginine	33	480	12
N-Hydroxyhomo-L-arginine	146	410	2.8
N-Hydroxynor-L-arginine	>1500	[a]	[a]

[a] Not published. Very limited and incomplete information about physico-chemical properties of *N*-hydroxy-L-arginine and its homologues is available. [b] No activity.

inhibitor, *S*-(2-boronoethyl)-L-cysteine (Fig. 3, Table II), supports a role of arginase in regulation of NO biosynthesis. A schematic diagram of the hypothesized involvement of arginase in the regulation of NO-mediated vasodilation is in Fig. 4.

TABLE II
A comparison of some arginase inhibitors[34,41,42]

Inhibitor	RLA IC_{50}, μmol/l
L-Arginine	6000[a]
Homo-L-arginine	60000
Nor-L-arginine	[b]
N-Hydroxy-L-arginine	200
N-Hydroxyhomo-L-arginine	20
N-Hydroxynor-L-arginine	1
BEC	0.4–0.6[c]
ABH	0.8

RLA, rat liver arginase; ABH, (2*S*)-amino-6-boronohexanoic acid; BEC, *S*-(2-boronoethyl)-L-cysteine; IC_{50}, concentration causing 50% inhibition. [a] K_m value for hydrolysis of L-Arg by RLA. [b] Not published. [c] Arginase inhibition constant in human smooth muscle.

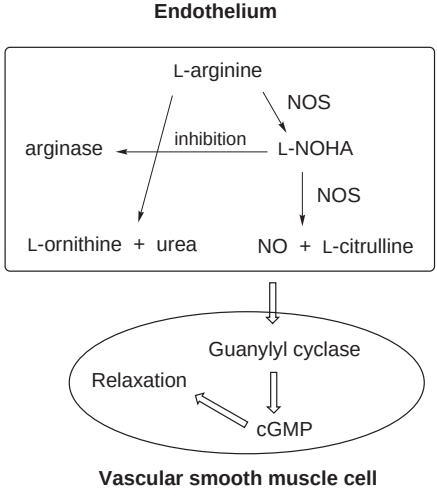


FIG. 4
Schematic representation of the involvement of arginase in the regulation of NO-mediated vasorelaxation[37]

5. CONCLUSION

NOHA is not only an intermediate of NOS catalyzed conversion of L-arginine to NO but it can be also converted to NO apparently by some other metabolic pathways independent of NOS. Available NOHA homologues could be useful in affecting competition between NOS and arginase for L-arginine. Homo-NOHA is a good substrate for NOS and does not affect arginase activity whereas nor-NOHA is not a substrate for NOS but it is a very good arginase inhibitor. NOHA and its homologues may be useful tools for characterization of NOS independent pathways to NO which are apparently multiple. They may also offer a novel approach for treating vascular diseases caused by impaired endothelial NOS activity.

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