Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 516 (2005) 260 – 267

www.elsevier.com/locate/ejphar

N^{ω}-hydroxy-L-arginine homologues and hydroxylamine as nitric oxide-dependent vasorelaxant agents

Petra Beranova[a], Karel Chalupsky[a], Andrei L. Kleschyov[b], Christa Schott[a], Jean-Luc Boucher[c], Daniel Mansuy[c], Thomas Munzel[b], Bernard Muller[a], Jean-Claude Stoclet[a,*]

[a]*Pharmacology and Physico-Chemistry, Centre National de la Recherche Scientifique and University Louis Pasteur of Strasbourg, Unité Mixte de Recherche 7034, Illkirch, France*

[b]*Second Medical Department, Johannes Gutenberg University, Mainz, Germany*

[c]*Laboratory of Pharmacological and Toxicological Biochemistry, Centre National de la Recherche Scientifique and University René Descartes, Unité Mixte de Recherche 8601, Paris, France*

Received 11 April 2005; accepted 14 April 2005

Abstract

Endothelium-independent relaxant activities of N^{ω}-hydroxy-L-arginine (L-NOHA) homologues and hydroxylamine, a possible intermediate in nitric oxide (NO) formation, were examined in rat aortic rings. Addition of one $-CH_2-$ group to the $-(CH_2)_x-$ chain between the α-amino acid and the hydroxyguanidine group ($x=4$) almost abolished–while deletion of one or two $-CH_2-$ ($x=1$ or 2) enhanced–the relaxant activity of L-NOHA homologues. N^{ω}-hydroxy-nor-L-arginine- ($x=2$) and hydroxylamine-induced relaxations were blunted by a NO scavenger and by inhibitors of the guanylyl cyclase pathway, but not by NO synthase or cytochrome P_{450} inhibitors (except 7-ethoxyresorufin). However, aortic NO formation was detected (using electron paramagnetic resonance) in the presence of concentrations of these compounds higher than those producing relaxation. These findings support the view that endothelium-independent vasorelaxations induced by both L-NOHA homologues with a required chain length $x \leq 3$ and hydroxylamine are mediated by NO-dependent activation of guanylyl cyclase, through a 7-ethoxyresorufin-inhibited mechanism.

Keywords: N^{ω}-hydroxy-L-arginine homologue; Hydroxylamine; Nitric oxide release; Vasorelaxation; Electron paramagnetic resonance

1. Introduction

N^{ω}-hydroxy-L-arginine (L-NOHA), the stable intermediate in nitric oxide (NO) synthase-catalyzed oxidation of L-arginine to NO and L-citrulline, has vasorelaxant properties (Wallace et al., 1991; Zembowicz et al., 1992; Abdul-Hussain et al., 1996). Its *N*-hydroxyguanidine group can be oxidized not only by NO synthase (Stuehr et al., 1991; Klatt et al., 1993) but also by other oxygenases, leading to formation of nitrogen oxides (Boucher et al., 1992; Jousserandot et al., 1998; Caro et al., 2001). In addition, L-NOHA is an arginase inhibitor (Boucher et al., 1994). The latter property might contribute to enhance NO synthase-dependent NO formation by increasing the enzyme substrate level in cells. However, it has been reported that L-NOHA and other non-amino-acid compounds bearing a C=NOH function can also produce NO synthase-independent relaxation in endothelium-denuded rat aortic rings, and that this relaxation involves the NO/guanylyl cyclase pathway (Vetrovsky et al., 2002). A NO synthase-independent pathway for NO production in blood vessels might be used to reduce the deleterious consequences of impaired endothelial NO synthase activity, which is associated with increased cardiovascular risk (for review, see Gewaltig and Kojda, 2002).

* Corresponding author. Pharmacologie et Physico-Chimie, Faculté de Pharmacie, BP 60024, 74, route du Rhin, Illkirch 67401, France. Tel.: +33 390 24 42 46; fax: +33 390 24 43 13.

E-mail address: stoclet@pharma.u-strasbg.fr (J.-C. Stoclet).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.04.021

The structure of L-NOHA comprises a hydroxyguanidine function separated from the α-amino acid function by three $-CH_2-$ groups. A previous structure-vasorelaxant activity study of compounds with a C=NOH function has shown that nonsubstituted hydroxyguanidine was practically inactive and that L-NOHA was more active than other non-amino-acid-substituted hydroxyguanidines (Vetrovsky et al., 2002). These findings suggest that the presence of the α-amino acid function and the $(CH_2)_3$ chain is an important structural determinant of the vasorelaxant effect of L-NOHA. The structural determinants involved in the recognition of α-amino acids bearing a C=NOH function by NO synthase and arginase have been studied by Moali et al. (1998, 2000), showing the importance of shortening or lengthening the $-CH_2-$ chain. However, influence of the chain length on the NO synthase-independent vasorelaxing effect of these amino acids has not been investigated as yet. Here endothelium-independent vasorelaxation produced by L-NOHA homologues with a $-(CH_2)_x-$ chain length where x varied from 1 to 4, was investigated in rat aortic rings, and involvement in the relaxation of the NO/guanylyl cyclase pathway was studied in the same tissue, using the most active homologue.

Like L-NOHA, hydroxylamine has been proposed as a possible intermediate in the oxidative conversion of L-arginine to NO by endothelial NO synthase (DeMaster et al., 1989; Thomas and Ramwell, 1989; Schmidt et al., 1990). Also like L-NOHA, it relaxes smooth muscle cells, and it is believed to do so through intracellular release of NO (see review by Waldman and Murad, 1987). Therefore hydroxylamine formation might also be an intermediate step in the NO synthase-independent conversion of L-NOHA to NO. In the present work, the involvement of NO in the endothelium-independent relaxant effect of this compound was also investigated, and its pharmacological profile was compared to the one of the most active L-NOHA homologues.

2. Materials and methods

2.1. Animals and thoracic aorta preparation

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (agreement no. B 67900, given by French authorities). Thoracic aortae were removed from male Wistar rats (11–14 weeks old, 300–380 g, bred from genitors provided by Iffa Credo), as described previously by Vetrovsky et al. (2002) (organ bath studies) and Chalupsky et al. (2004) (NO formation experiments). The vessels were cleaned of connective and fat tissues in Krebs solution (composition in mM: NaCl 119; KCl 4.7; $MgSO_4$ 1.17; $CaCl_2$ 1.25; KH_2PO_4 1.18; $NaHCO_3$ 25; glucose 11), and different rings were obtained from each aorta. Unless otherwise indicated, the endothelium was removed by gentle rubbing of the intimal surface of the rings with curved forceps.

Because the effects of L-NOHA homologues could vary from one experiment to the other, when performed at different times on aortic rings from different rats (Vetrovsky et al., 2002), comparisons between the homologues, or in the presence or absence of inhibitors, were performed in simultaneously run experiments using rings from the same rats.

2.2. Organ bath studies

For isometric tension recordings, endothelium-denuded rings (3–5 mm long) were mounted in organ chambers filled with Krebs solution (37 °C) bubbled with 95% O_2/5% CO_2, under a passive tension of 2 g. After equilibration, they were pre-contracted with noradrenaline (1 μM). Acetylcholine (1 μM) was subsequently added to verify the absence of functional endothelium. Rings were then pre-contracted with 0.1 μM noradrenaline or phenylephrine, and L-NOHA or homologues were added in a cumulative manner. In some experiments, the arginase inhibitors L-valine (30 mM) and 2(*S*)-amino-6-boronohexanoic acid (ABH; 100 μM) were added to noradrenaline (0.1 μM)-precontracted rings. When used, other inhibitors (or their solvent) were added 15 min (unless otherwise indicated) before induction of pre-contraction by noradrenaline or phenylephrine. As previously reported (Vetrovsky et al., 2002), there was no difference in relaxation produced by compounds with a C=NOH function when one or the other agonist was used, but in the presence of some inhibitors, phenylephrine-induced contraction was more stable than noradrenaline-induced contraction. The used inhibitors were N^{ω}-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthase, 300 μM); 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, a cell-permeable scavenger of NO, 300 μM); 1*H*[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, an inhibitor of the activation of soluble guanylyl-cyclase by NO, 1 μM); Rp-8-bromo cyclic GMP monophosphorothioate (Rp-8-Br-cGMPS, an inhibitor of cyclic GMP-dependent protein kinase, 100 μM, added in the bath 1h before noradrenaline); proadifen (10 μM) or 17-octadecynoic acid (17-ODYA, 30 μM), two nonselective inhibitors of cytochrome P_{450} added 30 min before phenylephrine; or 7-ethoxyresorufin (7-ER), a selective inhibitor of cytochrome P_{450} of the 1A family and of NADPH-dependent reductases (3 μM). ODQ (1 μM) was also added at the end of some relaxation experiments.

2.3. NO formation

Vascular NO formation was assessed in aortic rings without endothelium (unless otherwise indicated), using electron paramagnetic resonance spin trapping with colloid Fe-diethyldithiocarbamate (DETC) (Kleschyov et al., 2000). Briefly, rings (6–8 mm long) were incubated at 37 °C with colloid $Fe(DETC)_2$ (0.2 mM) for the indicated time, in the presence and absence of L-NOHA or homologue or hydroxylamine at the indicated concentrations. In some experiments, 7-ER (10 μM) was added with N^{ω}-hydroxy-nor-L-arginine (nor-L-NOHA) for 15 min. Tissues were then frozen in calibrated tubes and kept in liquid nitrogen until measurements. Electron paramagnetic resonance spectra were recorded on an MS100 spectrometer (Magnettech, Germany) under the following conditions: temperature 77 K, microwave frequency 9.34 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 0.5 mT, and time constant 100 ms. After measurements, the aortic tissues were dried and weighted. The relative amount of NO–$Fe(DETC)_2$ was determined by dividing the

amplitude of the third component of the characteristic signal (in arbitrary units) by the dry weight of the sample.

2.4. Drugs

Unless otherwise indicated, chemicals were purchased from Sigma and were dissolved in Krebs solution or MilliQ water (Millipore). L-NOHA and homologues (Moali et al., 2000) and ABH (Baggio et al., 1997) were synthesized as previously described. Sodium pentobarbital was purchased from Sanofi Santé Animale. PTIO was dissolved as a 10 mM solution in 50% ethanol. ODQ (from Tocris-Cookson) and 7-ER were dissolved as a 10 mM solution in 100% DMSO. All subsequent dilutions were performed in Krebs solution.

2.5. Data analysis

Results are expressed as mean±S.E.M. of n experiments. The relaxation is expressed as a percentage of the contraction induced by noradrenaline or phenylephrine (0.1 μM). Concentration–response curves were compared by multi-analysis of variance (MANOVA). Other statistical comparisons were performed with one-way ANOVA. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Influence of the chain length on the relaxant effect

The endothelium-independent concentration-dependent relaxant effects of L-NOHA homologues are shown in Fig. 1. The increase in the chain length from three (L-NOHA) to four (homo-L-NOHA) –CH_2– groups dramatically decreased, while the reduction of the number of –CH_2– groups to two (nor-L-NOHA) or one (dinor-L-NOHA) enhanced the relaxant effect. By contrast with L-NOHA and shorter homologues, L-valine (30 mM) and ABH (100 μM), two inhibitors of arginase, failed to reduce the aortic tone elicited by noradrenaline (not shown).

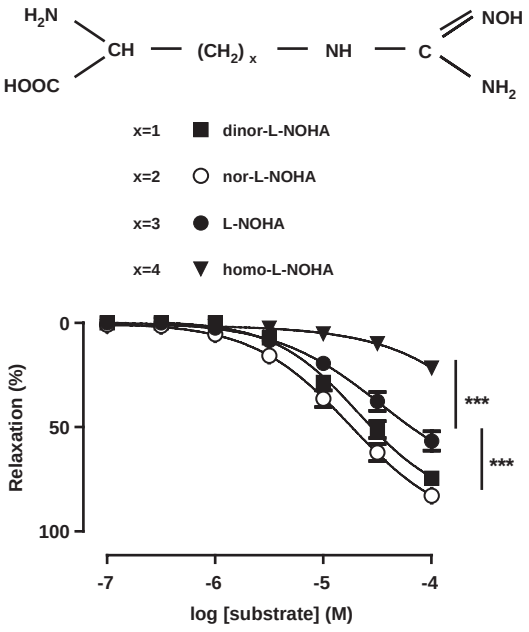

Fig. 1. Structure of L-NOHA and L-NOHA homologues (top), and their concentration–effect curves (bottom) in endothelium-denuded rat aortic rings pre-contracted with noradrenaline (0.1 μM). Results are expressed as mean±S.E.M. of 9 (homo-L-NOHA), 12 (L-NOHA), 4 (dinor-L-NOHA), and 8 (nor-L-NOHA) experiments. ***Significant difference ($P<0.001$) compared with L-NOHA.

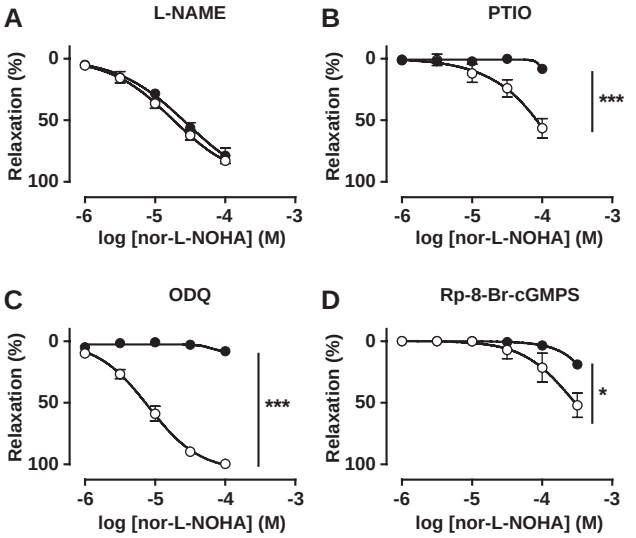

Fig. 2. Relaxant effect of nor-L-NOHA in noradrenaline (0.1 μM; A, C, and D) or phenylephrine (0.1 μM; B) pre-contracted endothelium-denuded rat aortic rings, in the absence (controls, open symbols) or in the presence (closed symbols) of: (A) L-NAME, 300 μM ($n=5$; controls, $n=4$); (B) PTIO, 300 μM ($n=6$; controls, $n=6$); (C) ODQ, 1 μM ($n=4$; controls, $n=4$); or (D) Rp-8-Br-cGMPS, 100 μM ($n=5$; controls, $n=5$). Significant differences with controls: *$P<0.05$, ***$P<0.001$.

3.2. Involvement of the NO/cyclic GMP pathway

In subsequent experiments, nor-L-NOHA (Fig. 2), one of the two most active homologues, was compared with hydroxylamine (Fig. 3). Hydroxylamine was about 100 times more active than nor-L-NOHA in producing aortic relaxation. As in the case of L-NOHA (Vetrovsky et al., 2002), the relaxations caused by both nor-L-NOHA and hydroxylamine were unchanged in the presence of the NO synthase inhibitor L-NAME (Figs. 2A and 3A), but they were blunted by the NO scavenger PTIO (Figs. 2B and 3B), by the inhibitor of NO-induced activation of guanylyl cyclase ODQ (Figs. 2C and 3C), and by the inhibitor of cyclic GMP-dependent protein kinase Rp-8-Br-cGMPS (Figs. 2D and 3D). Furthermore, addition of ODQ to the bath at the end of relaxation experiments with both nor-L-NOHA and hydroxylamine fully restored contraction to the level initially produced by noradrenaline (Fig. 4), as previously reported with L-NOHA (Vetrovsky et al., 2002).

3.3. Formation of NO

Representative electron paramagnetic resonance signals obtained in aortic rings incubated for 30 min in the presence of $Fe(DETC)_2$, a spin trap specific for NO, are shown in Fig. 5. A characteristic NO signal was seen in rings with endothelium (Fig. 5A and B). This signal was markedly enhanced in rings incubated in the presence of the calcium ionophore A23187 (calcimycine;

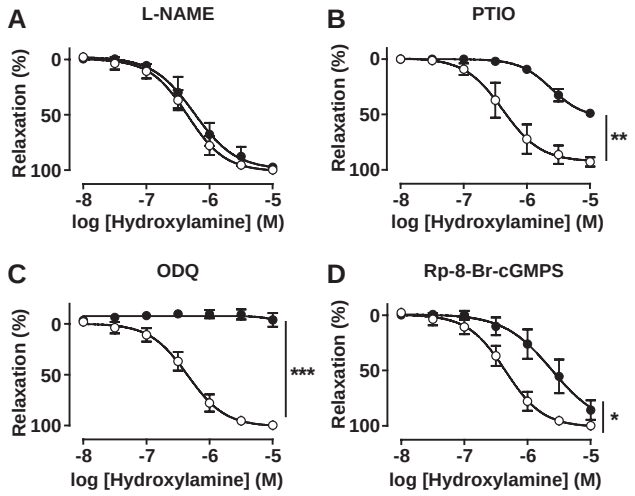

Fig. 3. Relaxant effect of hydroxylamine in noradrenaline (0.1 μM; A, C, and D) or phenylephrine (0.1 μM; B) pre-contracted endothelium-denuded rat aortic rings, in the absence (controls, open symbols) or in the presence (closed symbols) of: (A) L-NAME, 300 μM ($n=4$; controls, $n=6$); (B) PTIO, 300 μM ($n=3$; controls, $n=3$); (C) ODQ, 1 μM ($n=4$; controls, $n=6$); or (D) Rp-8-Br-cGMPS, 100 μM ($n=5$; controls, $n=6$). Significant differences with controls: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Fig. 5B). By contrast, no NO signal was seen in endothelium-denuded control rings (Fig. 5C). These data show that sensitivity of the used technique allowed to detect not only NO formation induced by an endothelium-dependent relaxing agent, but also basal endothelial NO production.

In the same experimental conditions, incubation with up to 3 μM hydroxylamine or 100 μM L-NOHA or nor-L-NOHA (Fig. 5D, E, and F, respectively) did not induce any detectable NO signal in endothelium-denuded rings. However, a significant increase in signal amplitude over basal level (i.e., noise) was found in rings exposed for 1 h to 1 mM nor-L-NOHA (Fig. 6) or 10 μM hydroxylamine (not shown), demonstrating significant NO formation in the latter condition.

3.4. Involvement of various mechanisms

Various mechanisms have been proposed to explain NO synthase-independent formation of NO from the hydroxyguanidine function. These include oxidation by cytochromes P_{450} (Renaud et al., 1993; Jousserandot et al., 1998). However, neither proadifen nor 17-ODYA (two nonselective inhibitors of the known cytochrome P_{450} isoforms) inhibited the nor-L-NOHA relaxation (Fig.

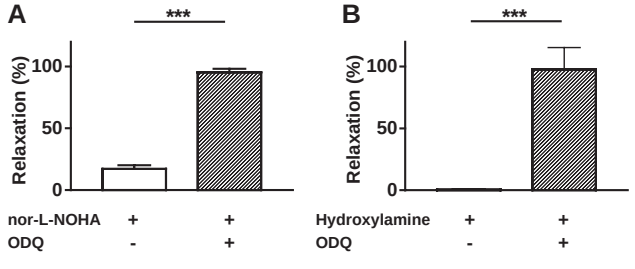

Fig. 4. Restoration of the vascular tone by addition of ODQ (1 μM) in rat aortic rings exposed to phenylephrine (0.1 μM) and nor-L-NOHA (100 μM, $n=7$; A) or hydroxylamine (10 μM, $n=6$; B). Significant differences: $***P<0.001$.

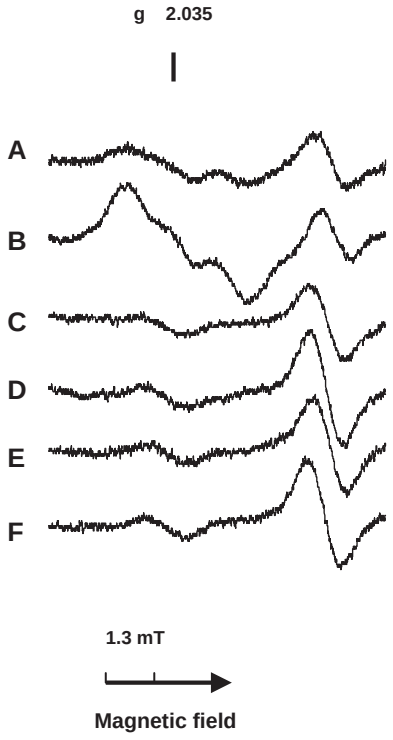

Fig. 5. Representative traces (of three experiments) of electron paramagnetic resonance spin trapping of NO in rat aortic rings with (A and B) or without (C–F) endothelium, incubated with 200 μM Fe $(DETC)_2$ for 30 min in the following conditions: (A and C) control (solvent); (B) A23187, 1 μM; (D) hydroxylamine, 3 μM; (E) L-NOHA, 100 μM; (F) nor-L-NOHA, 100 μM.

7A and B). By contrast, 7-ER, which is a suicide substrate of the cytochrome P_{450} $1A_1$ isoform (Tassaneeyakul et al., 1993) but also an inhibitor of NADPH-dependent P_{450} reductases (Dutton et al., 1989) and other reductases (Jiang and Ichikawa, 1999), blunted the relaxant effect of nor-L-NOHA (Fig. 7C). In the presence of 7-ER (10 μM), there was no significant electron paramagnetic resonance-detected NO formation from nor-L-NOHA (1 mM) (Fig. 6).

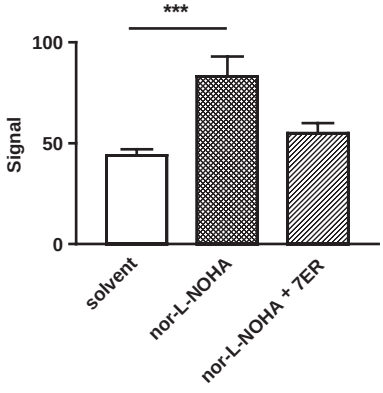

Fig. 6. Histograms showing the amplitude of the electronic paramagnetic signal (in arbitrary units at the magnetic field of the third component of the NO signal, per milligram of dry tissue) in endothelium-denuded aortic rings incubated for 1 h with colloid Fe$(DETC)_2$ (200 μM) and solvent (control, $n=14$), nor-L-NOHA (1 mM, $n=14$), or nor-L-NOHA plus 7-ER (10 μM, $n=5$). Significant differences with controls: $***P<0.001$.

Hydroxylamine relaxation exhibited a pharmacological profile comparable to that of nor-L-NOHA (Fig. 7D–F), in that it was inhibited by 7-ER, but neither by proadifen nor by 17-ODYA. However, proadifen and 17-ODYA even significantly potentiated the relaxant effect of hydroxylamine.

4. Discussion

The above findings show the importance of the length of $-CH_2-$ chain between the α-amino acid and the hydroxyguanidine function for the endothelium-independent vasorelaxant effect of L-NOHA amino acid homologues. They support the hypothesis that NO or a NO-related compound formed from these amino acids by a NO synthase-independent mechanism is involved in the activation of the soluble guanylyl cyclase and relaxation caused by these amino acids, although NO formation could be detected by electron paramagnetic resonance at concentrations higher than those producing relaxation. In addition, they show that hydroxylamine is a more potent endothelium-independent relaxant compound than L-NOHA homologues in rat aortic rings, but that it produces a qualitatively comparable effect with respect to involvement of the NO/guanylyl cyclase pathway and to pharmacological profile.

The influence of the chain length on vasorelaxant properties is in striking contrast with its impact on the substrate specificity of NO synthases: these enzymes favour NO formation from molecules such as L-NOHA and homo-L-NOHA, bearing a hydroxyguanidine function separated from the Cα carbon by three or four $-CH_2-$ groups. As homo-L-arginine had practically no effect in endothelium-denuded aortic rings, this compound was not further studied here. By contrast, reducing the chain length to two (nor-L-NOHA) or one (dinor-L-NOHA) results in relaxant compounds, which are nevertheless very poor NO synthase substrates (Moali et al., 1998). Consistent with these observations, L-NAME did not produce any inhibition of relaxations caused by nor-L-NOHA (this study) or L-NOHA in endothelium-denuded rat aorta (Vetrovsky et al., 2002). Thus, NO synthase, which might be contained in the vessel adventitia/media layers, did not account for relaxation caused by these amino acids.

Another known consequence of reducing the chain length from three to two $-CH_2-$ is the marked increase of the inhibitory potency of the amino acids towards arginase, with nor-L-NOHA being 20 times more potent than L-NOHA in this respect (Moali et al., 2000). By decreasing L-arginine consumption in cells, the inhibition of arginase might enhance NO synthase-mediated NO production. However, this mechanism was unlikely to be involved in aortic relaxation due to a low or absent NO synthase activity in endothelium-denuded rat aorta. In agreement with this conclusion is the lack of effect of L-NAME and two arginase inhibitors, L-valine and ABH (Baggio et al., 1999), in the present study. The finding, that dinor-L-NOHA was equipotent to nor-L-NOHA as a relaxant agent whereas it was 40 times less potent as an arginase inhibitor (Moali et al., 2000), is also in keeping with this conclusion.

Involvement of the NO/guanylyl cyclase pathway was further studied using nor-L-NOHA. Relaxation induced by this compound was blunted by the NO scavenger PTIO, by the inhibitor of NO-induced soluble guanylyl cyclase activation ODQ, and by the G kinase inhibitor Rp-8-Br-cGMPS. Similar findings were previously reported using L-NOHA (Vetrovsky et al., 2002). Altogether these data support the view that the mechanisms of aortic relaxation caused by the two homologues were identical, and they provide indirect evidence that release of NO or a NO-related compound activating the soluble guanylyl cyclase was implicated. However, a rather high concentration of nor-L-NOHA (1 mM) had to be used to induce significant electron paramagnetic resonance detectable NO formation. Similarly, high concentrations of non-amino-acid compounds with a C=NOH function were required to demonstrate NO formation in rat aortic rings, using the same technique and experimental conditions (Chalupsky et al., 2004). Also, using the same technique as here, Kleschyov et al. (2003) recently reported that, in contrast to endothelium-dependent

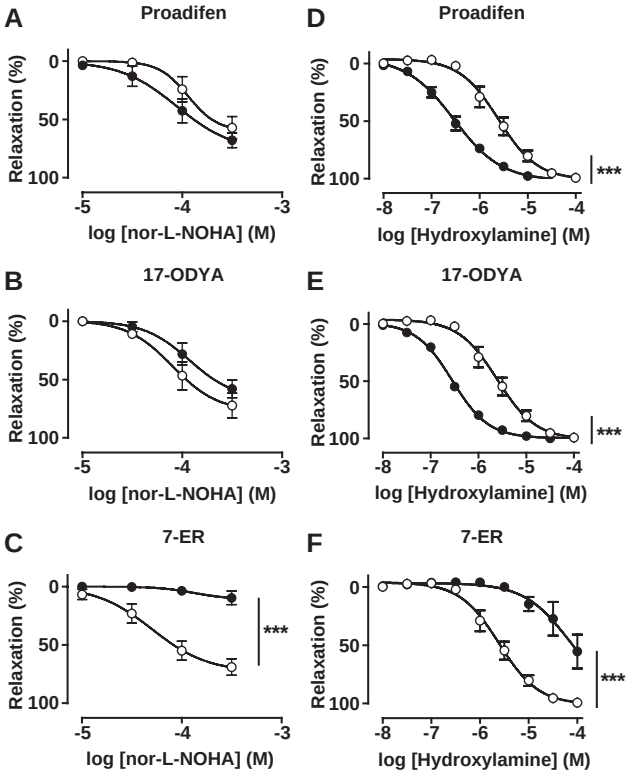

Fig. 7. Effects of cytochrome P_{450} inhibitors (A and D: proadifen, 10 μM), 17-octadecynoic acid (B and E: 17-ODYA, 30 μM), and 7-ethoxyresorufin (C and F: 7-ER, 3 μM) on vasorelaxations elicited by nor-L-NOHA (A–C) and hydroxylamine (D–F) in endothelium-denuded rat aortic rings pre-contracted with phenylephrine (0.1 μM). In each case, the data were obtained in the absence (open symbols, $n=5$) or in the presence of an inhibitor (closed symbols, $n=5$). Significant difference with controls: ***$P<0.001$.

vasodilators, nitroglycerin at vasorelaxant concentrations failed to produce any electron paramagnetic resonance-detectable free NO in isolated blood vessels. This is in striking contrast with the finding that, in the same tissue, the technique allowed to detect basal endothelial NO production and that a marked NO signal was induced by the ionophore A23187, an endothelium-dependent relaxant agent. Thus, the technique was able to detect endothelium-derived NO associated with relaxation. The finding that it was unable to detect NO when the vessel was exposed to relaxant concentrations of compounds acting by an endothelium-independent mechanism may indicate that the probe was unable to detect intracellular minute amounts of the drug-derived NO that were nevertheless sufficient and appropriately located to activate the soluble guanylyl cyclase in vascular smooth muscle cells. Alternatively, activation of the guanylyl cyclase might occur without mediation of the free radical NO, as it has been suggested in the case of nitroglycerin (Kleschyov et al., 2003).

The endothelium- and NO synthase-independent mechanism by which cleavage of C=NOH bonds may release NO or a NO-related activator of guanylyl cyclase in vascular tissues is unknown. The present data show that 7-ER is able to inhibit the relaxant effect of nor-L-NOHA, as it inhibits the ones of L-NOHA and other non-amino-acid compounds bearing a C=NOH function, again supporting the view that similar mechanisms are involved in relaxation (Vetrovsky et al., 2002; Chalupsky et al., 2004). By contrast, the relaxant effect of nitroglycerin in endothelium-denuded rat aortic rings was not modified by 7-ER, suggesting the implication of different mechanism (Chalupsky et al., 2004).

Oxidation of C=NOH functions by superoxide or other reactive oxygen species generated by uncoupled oxygenases, including cytochromes P_{450} or other oxygen radical-generating systems, might result in NO formation in tissues (Sennequier et al., 1995; Jousserandot et al., 1998; Caro et al., 2001). Nonselective inhibitors of cytochromes P_{450} failed to inhibit the relaxant effects of nor-L-NOHA (this study) and other compounds bearing a C=NOH function in the rat aorta (Vetrovsky et al., 2002; Chalupsky et al., 2004). Finally, 7-ER and diphenyliodonium, an inhibitor of NAD(P)H-dependent enzymes, were the only compounds found to inhibit relaxation induced by L-NOHA (Vetrovsky et al., 2002). 7-ER has been reported to be not only a suicide substrate of cytochrome P_{450} $1A_1$ (Tassaneeyakul et al., 1993) but also an inhibitor of NADPH-dependent reductases (Jiang and Ichikawa, 1999). This leads to the hypothesis that formation of NO from L-NOHA homologues may rely on 7-ER-sensitive cytochrome(s) P_{450} of the 1A family or NADPH-dependent reductase(s) activity in this tissue. These enzyme(s) might produce a yet unknown compound able to either directly or indirectly (through subsequent NO formation) activate guanylyl cyclase.

There are striking similitudes between the data obtained with L-NOHA, nor-L-NOHA, and hydroxylamine: involvement of the NO/guanylyl cyclase pathway in the mechanism of relaxation, absence of detectable electronic paramagnetic resonance NO signal at concentrations that produced relaxation, and inhibition of relaxation by 7-ER but neither by NO synthase nor by nonspecific cytochrome P_{450} inhibitors. One difference between hydroxylamine and the homologues is that relaxation produced by the former, but not the latter, was potentiated by both proadifen and 17-ODYA. Existence of this difference might suggest a different mechanism of relaxant action. However, there is another possibility that could be related to the instability of hydroxylamine in the organ bath experimental conditions. Under these conditions, hydroxylamine should be easily oxidized by various enzymes or reactive species such as superoxide anion (Schmidt et al., 1990) into various nitrogen oxides, such N_2O, NO, NO_2, NO_2^-, and NO_3^-. Potentiation of the relaxing effects of hydroxylamine by proadifen and 17-ODYA, which are nonselective inhibitors of cytochromes P_{450}, would suggest that most cytochromes P_{450} oxidatively transform hydroxylamine with very minor formation of NO compared to the other nitrogen oxides. By contrast, inhibition of the relaxing effects of hydroxylamine by 7-ER would indicate that the enzymes specifically inhibited by 7-ER [i.e., cytochromes P_{450} of the 1A family (Tassaneeyakul et al., 1993) and NADPH-dependent P_{450} reductases (Dutton et al., 1989)] catalyze the transformation of hydroxylamine into a guanylyl cyclase-activating compound.

As the concentrations of hydroxylamine that produced relaxation were about 100 times lower than the ones of L-NOHA homologues, release of small amounts of hydroxylamine from these homologues remains a possible intermediate step in the subsequent formation of NO or a NO-like compound. The mechanism of hydroxylamine-induced vasorelaxation may involve the inhibition of K^+ channels by NO-dependent and independent mechanisms (Huang, 1998; Tang et al., 2005). In cell-free system (Nicholls, 1964; Craven et al., 1979) or in the presence of neutrophils (Klink et al., 2001) or in the brain (Ohta et al., 1997), hydroxylamine is converted by catalase to NO and superoxide anion, in the presence of hydrogen peroxide. However, the catalase inhibitor 1,2,4-aminotriazole failed to inhibit hydroxylamine-induced relaxation in rat duodenum (Correira et al., 2000) and both L-NOHA- and hydroxylamine-induced relaxation in rat aorta (unpublished personal findings), suggesting that a catalase-mediated reaction was not involved in smooth muscle relaxation. Rather, the present finding that the NO/guanylyl cyclase-dependent hydroxylamine relaxation was inhibited by 7-ER suggests that either NADPH-dependent reductase(s) or cytochrome(s) P_{450} of the 1A family were involved in the formation of a guanylyl cyclase-activating compound from hydroxylamine, as well as from L-NOHA homologues with a chain length ≤ 3 $-CH_2-$. Further experiments are needed to clarify this point.

Whatever the mechanism of release of NO or a NO-related activator of soluble guanylyl cyclase, the finding that

nor-L-NOHA, L-NOHA, other non-amino-acid derivatives with a C=NOH function, and hydroxylamine can activate the pathway without release of excessive amounts of NO within vascular tissues is of interest. This might be important for therapeutic applications such as vascular protection or treatment of angina pectoris, as the release of large quantities of NO in tissues may be deleterious.

In conclusion, this study shows the importance of the presence and the length of the $-(CH_2)_x-$ chain separating the hydroxyguanidine function from the α-amino acid function for the NO synthase-independent vasorelaxant effect of L-NOHA homologues. It emphasises that structural determinants for aortic relaxation are different from those previously reported for recognition by NO synthase and by arginase. In addition, the present findings obtained with nor-L-NOHA and hydroxylamine lend further support to the hypothesis that endothelium-independent aortic relaxation produced by amino acids bearing a hydroxyguanidine function implicates the NO/guanylyl cyclase pathway, with possible formation of hydroxylamine as intermediate step, and that a 7-ER-inhibited, NADPH-dependent reductase might be involved in the mechanisms leading to activation of guanylyl cyclase. Finally, comparison of the data for relaxation and electron paramagnetic resonance studies suggests that, as recently reported for nitroglycerin but not for some other NO donors, relaxation elicited by these compounds is not associated with a large increase of vascular NO content. Such a property might be important for therapeutic applications in vascular diseases with impaired endothelial NO synthase activity.

Acknowledgements

This work was partially supported by grant 00967ZD from the Barrande Program of the French Ministry of Forein Affairs. P.B. and K.C. were recipients of a fellowship from the French Embassy in the Czech Republic.

References

Abdul-Hussain, M.N., Jia, Y.L., Hussain, S.N.A., 1996. Mechanisms mediating the vasodilatory effects of N^{ω}-hydroxy-L-arginine in coronary arteries. Eur. J. Pharmacol. 305, 155–161.

Baggio, R., Elbaum, D., Kanyo, Z.F., Caroll, P.J., Cavalli, R.C., Ash, D.E., Christianson, D.W., 1997. Inhibition of Mn^{2+} arginase by borate leads to the design of a transition state analogue inhibitor, 2(*S*)-amino-6-borohexanoic acid. J. Am. Chem. Soc. 119, 8107–8108.

Baggio, R., Emig, F.A., Christianson, D.W., Ash, D.E., Chakder, S., Rattan, S., 1999. Biochemical and functional profile of a newly developed potent and isozyme-selective arginase inhibitor. J. Pharmacol. Exp. Ther. 290, 1409–1416.

Boucher, J.L., Genet, A., Vadon, S., Delaforge, M., Mansuy, D., 1992. Formation of nitrogen oxides and citrulline upon oxidation of *N*-omega-hydroxy-L-arginine by hemeproteins. Biochem. Biophys. Res. Commun. 184, 1158–1164.

Boucher, J.L., Custot, J., Vadon, S., Delaforge, M., Lepoivre, M., Tenu, J.P., Yapo, A., Mansuy, D., 1994. N^{ω}-hydroxy-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver macrophage arginase. Biochem. Biophys. Res. Commun. 203, 1614–1621.

Caro, A.A., Cederbaum, A.I., Stoyanovsky, D.A., 2001. Oxidation of the ketoxime acetoxime to nitric oxide by oxygen radical-generating systems. Nitric Oxide 5, 413–424.

Chalupsky, K., Lobysheva, I., Nepveu, F., Gadea, I., Beranova, P., Entlicher, G., Stoclet, J.C., Muller, B., 2004. Relaxant effect of oxime derivatives in isolated rat aorta: role of nitric oxide (NO) formation in smooth muscle. Biochem. Pharmacol. 67, 1203–1214.

Correira, N.A., Oliviera, R.B., Ballejo, G., 2000. Pharmacological profile of nitrergic nerve-, nitric oxide-, nitrosoglutathione- and hydroxylamine-induced relaxations of the rat duodenum. Life Sci. 66, 709–717.

Craven, P.A., DeRubertis, F.R., Pratt, D.W., 1979. Electron spin resonance study of the role of NO catalase in the activation of guanylate cyclase by NaN_3 and NH_2OH. J. Biol. Chem. 254, 8213–8222.

DeMaster, E.G., Raij, L., Archer, S.L., Weir, E.K., 1989. Hydroxylamine is a vasorelaxant and a possible intermediate in the oxidative conversion of L-arginine to nitric oxide. Biochem. Biophys. Res. Commun. 163, 527–533.

Dutton, D.R., Reed, G.A., Parkinson, A., 1989. Redox cycling of resorufin catalyzed by rat liver microsomal NADPH–cytochrome P_{450} reductase. Arch. Biochem. Biophys. 268, 605–616.

Gewaltig, M.T., Kojda, G., 2002. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc. Res. 55, 250–260.

Huang, Y., 1998. Hydroxylamine-induced relaxation inhibited by K^+ channel blockers in rat aortic rings. Eur. J. Pharmacol. 349, 53–60.

Jiang, H.B., Ichikawa, Y., 1999. Neuronal nitric oxide synthase catalyzes the reduction of 7-ethoxyresorufin. Life Sci. 65, 1257–1264.

Jousserandot, A., Boucher, J.L., Henry, Y., Niklaus, B., Clement, B., Mansuy, D., 1998. Microsomal cytochrome P_{450} dependent oxidation of *N*-hydroxyguanidines, amidoximes, and ketoximes: mechanism of the oxidative cleavage of their C=N(OH) bond with formation of nitrogen oxides. Biochemistry 37, 17179–17191.

Klatt, P., Schmidt, K., Uray, G., Mayer, B., 1993. Multiple catalytic functions of brain nitric oxide synthase. Biochemical characterization, cofactor-requirement, and the role of *N*-omega-hydroxy-L-arginine as an intermediate. J. Biol. Chem. 268, 14781–14787.

Kleschyov, A.L., Mollnau, H., Oelze, M., Meinertz, T., Huang, Y., Harrison, D.G., Munzel, T., 2000. Spin trapping of vascular nitric oxide using colloid Fe(II)-diethyldithiocarbamate. Biochem. Biophys. Res. Commun. 275, 672–677.

Kleschyov, A.L., Oelze, M., Daiber, A., Huang, Y., Mollnau, H., Schulz, E., Sydow, K., Fichtlscherer, B., Mulsch, A., Munzel, T., 2003. Does nitric oxide mediate the vasodilator activity of nitroglycerin? Circ. Res. 93, e104–e112.

Klink, M., Swierzko, A., Sulowska, Z., 2001. Nitric oxide generation from hydroxylamine in the presence of neutrophils and in the cell free system. APMIS 109, 493–499.

Moali, C., Boucher, J.L., Sari, M.A., Stuehr, D.J., Mansuy, D., 1998. Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their *N*-omega-hydroxy derivatives, and *N*-omega-hydroxynor-L-arginine. Biochemistry 37, 10453–10460.

Moali, C., Brolo, M., Custot, J., Sari, M.A., Boucher, J.L., Stuehr, D.J., Mansuy, D., 2000. Recognition of α-amino acids bearing various C=NOH functions by nitric oxide synthase and arginase involves very different structural determinants. Biochemistry 39, 8208–8218.

Nicholls, P., 1964. The reactions of azide with catalase and their significance. Biochem. J. 90, 331–343.

Ohta, K., Rosner, G., Graf, R., 1997. Nitric oxide generation from sodium nitroprusside and hydroxylamine in brain. NeuroReport 8, 2229–2235.

Renaud, J.P., Boucher, J.L., Vadon, S., Delaforge, M., Mansuy, D., 1993. Particular ability of liver P450s to catalyze the oxidation of N^{ω}-hydroxy-L-arginine to citrulline and nitrogen oxides and occurrence in NO synthases of a sequence very similar to the heme binding sequence in P450s. Biochem. Biophys. Res. Commun. 192, 53–60.

Schmidt, H.H., Zernikow, B., Baeblich, S., Bohme, E., 1990. Basal and stimulated formation and release of L-arginine-derived nitrogen oxides from cultured endothelial cells. J. Pharmacol. Exp. Ther. 254, 591–597.

Sennequier, N., Boucher, J.L., Battioni, P., Mansuy, D., 1995. Superoxide anion efficiently performs the oxidative cleavage of C=NOH bonds of amidoximes and *N*-hydroxyguanidines with formation of nitrogen oxides. Tetrahedron Lett. 36, 6059–6062.

Stuehr, D.J., Kwon, N.S., Nathan, C.F., Griffith, O.W., Feldman, P.L., Wiseman, J., 1991. *N*-omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J. Biol. Chem. 266, 6259–6263.

Tang, G., Wu, L., Wang, R., 2005. The effect of hydroxylamine on KATP channels in vascular smooth muscle and underlying mechanisms. Mol. Pharmacol. 67, 1723–1731.

Tassaneeyakul, W., Birkett, D.J., Veronese, M.E., McManus, M.E., Tukey, R.H., Quattrochi, L.C., Gelboin, H.V., Miners, J.O., 1993. Specificity of substrate and inhibitor probes for human cytochromes P_{450} $1A_1$ and 1A2. J. Pharmacol. Exp. Ther. 265, 401–407.

Thomas, G., Ramwell, P.W., 1989. Vascular relaxation mediated by hydroxylamines and oximes: their conversion to nitrites and mechanism of endothelium dependent vascular relaxation. Biochem. Biophys. Res. Commun. 164, 889–893.

Vetrovsky, P., Boucher, J.L., Schott, C., Beranova, P., Chalupsky, K., Callizot, N., Muller, B., Entlicher, G., Mansuy, D., Stoclet, J.C., 2002. Involvement of NO in the endothelium-independent relaxing effects of *N*(omega)-hydroxy-L-arginine and other compounds bearing a C=NOH function in the rat aorta. J. Pharmacol. Exp. Ther. 303, 823–830.

Waldman, S.A., Murad, F., 1987. Cyclic GMP synthesis and function. Pharmacol. Rev. 39, 163–196.

Wallace, G.C., Gulati, P., Fukuto, J.M., 1991. N^{ω}-hydroxy-L-arginine: a novel arginine analog capable of causing vasorelaxation in bovine intrapulmonary artery. Biochem. Biophys. Res. Commun. 176, 528–534.

Zembowicz, A., Swierkosz, T.A., Southan, G.J., Hecker, M., Vane, J.R., 1992. Potentiation of the vasorelaxant activity of nitric oxide by hydroxyguanidine: implications for the nature of endothelium-derived relaxing factor. Br. J. Pharmacol. 107, 1001–1007.