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Biochemical Pharmacology 67 (2004) 1203–1214
Biochemical Pharmacology
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Relaxant effect of oxime derivatives in isolated rat aorta: role of nitric oxide (NO) formation in smooth muscle

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Received 15 September 2003; accepted 19 November 2003

Abstract

Various oxime derivatives were evaluated as nitric oxide (NO) donors in arteries. Relaxation of rat aortic rings was used for bioassay of NO production, and electron paramagnetic resonance spectroscopy for demonstration of NO elevation. In rings with or without endothelium or adventitia, hydroxyguanidine and hydroxyurea were almost inactive, whereas formamidoxime, acetaldoxime, acetone oxime, acetohydroxamic acid and formaldoxime elicited relaxation. Active compounds increased NO levels in endothelium-denuded rings. Formaldoxime was the most potent agent for both relaxation and NO elevation in aortic rings, and it also increased NO in human aortic smooth muscle cells. In endothelium-denuded rings, relaxation was inhibited by a NO scavenger (2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide) and by inhibitors of soluble guanylyl-cyclase (1*H*[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one) or cyclic GMP-dependent protein kinases (Rp-8-bromo cyclic GMP monophosphorothioate). Neither N^{ω}-nitro-L-arginine methylester (a NO synthases inhibitor) nor proadifen (a cytochrome P450 inhibitor) decreased the effect of oxime derivatives. However, 7-ethoxyresorufin (7-ER, an inhibitor of P4501A_1 which can also inhibit various NADPH-dependent reductases) abolished the relaxant effect of these compounds, without affecting the one of glyceryl trinitrate (GTN) or 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide. 7-ER also abolished formaldoxime-induced NO increase in aortic rings. In rings tolerant to GTN, formaldoxime-induced relaxation and NO elevation were not different from those obtained in control rings. In conclusion, some oxime derivatives release NO by 7-ER-sensitive pathways in aortic smooth muscle, thus eliciting vasorelaxation. Pathways of NO formation are likely distinct from NO synthases and from those responsible for GTN biotransformation. Oxime derivatives could be useful for NO delivery in arteries in which endothelial NO synthase activity is impaired.

Keywords: Electron paramagnetic resonance; 7-Ethoxyresorufin; Nitric oxide; Oxime derivatives; Rat aorta; Vasorelaxation

1. Introduction

NO is synthesized from L-arginine by NO synthases (NOS) in a two-step reaction [1,2]. The first step is the *N*-hydroxylation of one guanidino group of L-arginine, forming N^{ω}-hydroxy-L-arginine (L-NOHA). In the second step of the reaction, L-NOHA is metabolized into L-citrulline and NO. The synthesis L-NOHA from L-arginine is considered to be specific of NOS. However, the oxidative cleavage of the C=NOH bond of L-NOHA into NO or other nitrogen oxides can be catalyzed not only by NOS, but also by other hemoproteins, such as microsomal cytochromes P450 [3,4] or peroxidases [5]. NOS-independent pathways

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Abbreviations: DETC, diethyldithiocarbamate; DEA-NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; EPR, electron paramagnetic resonance; 7-ER, 7-ethoxyresorufin; GTN, glyceryl trinitrate; HASMC, human aortic smooth muscle cells; MEM, minimum essential medium; L-NA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; NO, nitric oxide; L-NOHA, N^{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; ODQ, 1*H*[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one; 17-ODYA, 17-octadecynoic acid; PHE, phenylephrine; PTIO, 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; Rp-8-Br-cGMPS, Rp-8-bromo cyclic GMP monophosphorothioate.

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doi:10.1016/j.bcp.2003.11.012

are also involved in the oxidative cleavage of the C=NOH bond of some synthetic compounds not bearing an alpha-amino acid function, like *N*-hydroxyguanidines, amidoximes and ketoximes [6–11].

In blood vessels, endothelial NOS-derived NO is a major protective factor. It prevents leukocyte adhesion and platelet adhesion/aggregation, and also inhibits proliferation and contraction of vascular smooth muscle cells [12,13]. Various pathological states (diabetes, hypercholesterolemia, atherosclerosis, etc.) are associated with impaired expression or activity of endothelial NOS (NOS-3) [14,15]. In these situations, the existence of NOS-independent pathways for NO production from exogenous substrates is of particular interest for restoration of NO levels. This can be obtained by administration of NO-donating agents, including organic nitrates, like GTN, whose biotransformation into NO or NO-related vasorelaxant species in blood vessels is independent of NOS activity [16,17]. Recently, it has been reported that L-NOHA and some synthetic non-alpha-amino acid compounds bearing a C=NOH bond (aromatic monosubstituted amidoximes and ketoximes) induced endothelium-dependent relaxation in the rat aorta [18]. Pharmacological evidence was obtained that the vasorelaxant effect of L-NOHA and 4-chlorobenzamidoxime was mediated by NO [18]. Although the metabolic pathways responsible for NO production from these compounds were not identified, the relaxant effect of L-NOHA and 4-chlorobenzamidoxime was inhibited by 7-ER [18]. 7-ER is not only a high affinity substrate and competitive inhibitor of cytochrome $P4501A_1$ [19] but it also interacts with NADPH-cytochrome P450 reductase, the redox partner for P450 [20] and with the reductase domain of structurally related NADPH-dependent enzymes, thus inhibiting their catalytic activity in a non-competitive manner [21].

In the present study, various non-aromatic substituted oxime derivatives were evaluated as NO donors in arteries. Compounds under investigation were formamidoxime, hydroxyguanidine, acetaldoxime, acetone oxime, hydroxyurea, acetohydroxamic acid and formaldoxime (see chemical structure in Fig. 1). Relaxation of rat aortic rings was used for bioassay of NO production. The role of the NO-cyclic GMP pathway in vasorelaxation and the pathways responsible for NO production were characterized using various pharmacological agents. Electron paramagnetic resonance (EPR) spectroscopy was also applied for demonstration of NO formation in arteries and smooth muscle cells. In some cases, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) and GTN were used for comparison, as prototype compounds which spontaneously release NO, or which are subjected to enzymatic biotransformation into NO or NO-related vasorelaxant species, respectively. Preliminary reports of this study have been presented as abstracts [22,23].

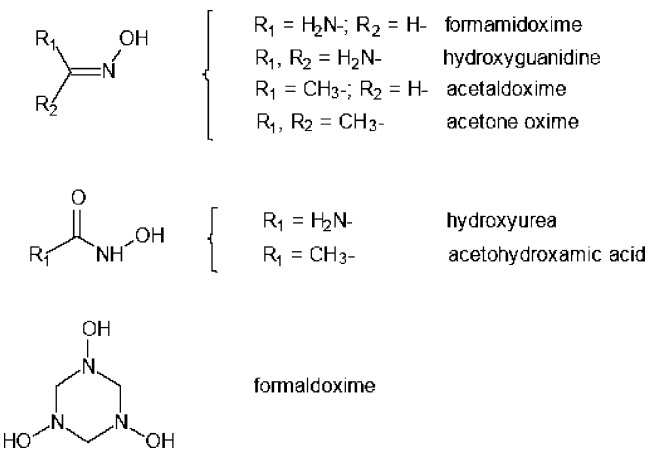

Fig. 1. Chemical structure of the compounds studied.

2. Materials and methods

2.1. Preparation of arteries

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 number B 67900, given by French authorities). Thoracic aorta were removed from male Wistar rats (11–14 weeks old, 300–380 g, bred from genitors provided by Iffa Credo) after anesthesia with pentobarbital (60 mg/kg, i.p.). It was 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). For most experiments, the endothelium was removed by gentle rubbing the intimal surface of the rings with curved forceps. In some cases, the adventitial layer was separated from the medial layer by microdissection. After careful cleaning of adherent adipose tissue and collateral vessels, adventitia/media could be clearly distinguished at the ends of aorta under magnification. The dissection was started from the arch-end of aorta by splitting of aortic wall with the use of two microsurgery forceps and gentle peeling of inverted adventitia toward distal end [24,25]. The intimal surface of the rings was rubbed to remove endothelial cells.

2.2. Contraction/relaxation experiments

For isometric tension recordings, rings (3–5 mm length) were mounted in organ chambers filled with Krebs solution (37°, under bubbling with 95% O_2/5% CO_2) under a passive tension of 2 g. After equilibration, they were precontracted with phenylephrine (PHE, 1 μM). Acetylcholine (1 μM) was subsequently added to verify the presence or absence of functional endothelium. Rings were considered to be denuded of functional endothelium when acetylcholine failed to induce a relaxant response. Endothelium was considered to be present when acetylcholine produced more than 50% relaxation. After washout,

the rings were precontracted either with 0.1 μM (rings without endothelium) or 0.3 μM PHE (rings with endothelium) in order to produce similar level of precontraction. When a steady-state level of contraction was obtained, oxime derivatives, GTN or DEA-NO, were added in a cumulative manner. When used, inhibitors (or their solvent) were added before PHE. The inhibitors used were 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 soluble guanylyl-cyclase by NO, 1 μM), Rp-8-bromo cyclic GMP monophosphorothioate (Rp-8-Br-cGMPS, an inhibitor of cyclic GMP-dependent protein kinases, 100 μM), 7-ER (2 μM), N^{ω}-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NOS, 300 μM), proadifen (also known as SKF 525a, an inhibitor of cytochromes P450, 30 μM), 17-octadecynoic acid (17-ODYA, a suicide inhibitor of cytochromes P450, 30 μM), or tranylcypromine (an inhibitor of monoamine oxidase and prostacyclin synthase, 30 μM). The concentrations of inhibitors which were used were equal or slightly above those mentioned in previous studies [18,26–30]. A standard incubation time of 30 min was chosen for most inhibitors. Based on our previous experiments with inhibitors of the NO-cyclic GMP pathway, incubation time was shorter (15 min) for ODQ and L-NAME or longer (45 min) for Rp-8-Br-cGMPS. In some experiments, endothelium-denuded rings were exposed to 100 μM GTN (or its solvent) or 1 mM formaldoxime for 1 hr. After several washouts (during at least 1 hr during which time the Krebs solution was replaced four times), rings were precontracted with PHE, and GTN, DEA-NO, or formaldoxime was subsequently added in a cumulative manner.

2.3. Human aortic smooth muscle cells (HASMC)

HASMC were obtained from Clonetics and cultured in MEM containing 10% fetal calf serum and antibiotics as previously described [31].

Experiments were performed with HASMC from passages 5 to 15, which were serum-deprived for 24 hr.

2.4. NO spin trapping and EPR spectroscopy

The NO content was assayed after formation of EPR-detectable [Fe(II)NO(diethyldithiocarbamate)$_2$] ([Fe(II)-NO(DETC)$_2$]) in endothelium-denuded rat aortic rings (6–8 mm length) or HASMC (10^6 in suspension). Preparations were exposed for 1 hr at 37° to oximes derivatives and colloid [Fe(II)(DETC)$_2$] (0.5 mM) in Krebs solution containing bovine albumin (final concentration 25 mg/mL) and HEPES (15 mM, pH 7.4) instead of $NaHCO_3$ [27,32]. In some experiments, rings were first exposed to 100 μM GTN for 1 hr and carefully washed before addition of formaldoxime (1 mM) and [Fe(II)(DETC)$_2$]. In another set of experiments, 7-ER (2 or 10 μM) was added 30 min before formaldoxime (1 mM) and [Fe(II)(-DETC)$_2$]. Tissues or cells were then rapidly frozen in calibrated tubes (0.3 mL) and kept in liquid nitrogen until EPR measurements. EPR spectra were recorded on an MS100 spectrometer (Magnettech) 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, time constant 100 ms. After EPR measurements, the aortic tissues were dried and weighted and the relative [Fe(II)NO(-DETC)$_2$] concentrations were determined by dividing the third component amplitude of the three-line EPR signal by the weight of the dried sample. For cells, the amplitude of the EPR signal was divided by the volume of the sample.

2.5. Determination of NO_x

Concentrations of NO_2^- were measured using Griess reagent, after 1-hr incubation of the compounds in Krebs solution at 37°. Concentrations of NO_3^- were evaluated after conversion of NO_3^- into NO_2^- using nitrate reductase followed by NO_2^- determination with Griess reagent [33]. The detection limit for NO_x was about 1 μM.

2.6. Drugs and reagents

Formaldoxime (formaldoxime trimer, or triformoxime) hydrochloride was purchased from Fluka, hydroxyurea from Serva. Acetone oxime, formamidoxime hydrochloride, acetohydroxamic acid, hydroxyguanidine, acetaldoxime (acetaldehyde oxime) were obtained from Sigma-Aldrich. Unless otherwise indicated, other chemicals were purchased from Sigma and were dissolved in Krebs solution or MilliQ water (Millipore). Sodium pentobarbital was purchased from Sanofi Santé Animale. GTN (1.5 mg/mL in propyleneglycol, LENITRAL®) was obtained from Besins International. DEA-NO (from Alexis Corporation) was made as a 10 mM stock solution in NaOH (10 mM). PTIO was dissolved as a 10 mM solution in 50% ethanol. 17-ODYA was dissolved as 10 mM stock solution in absolute ethanol. ODQ (from Tocris-Cookson) was dissolved as 10 mM solution in 100% DMSO, as well as 7-ER and proadifen.

2.7. Statistical analysis

Results are expressed as mean ± SE mean of N experiments. The relaxant effect was expressed in percentage of contraction, 100% being the tone induced by PHE. The EC_{50} values of the compounds (concentration that produced 50% relaxation of precontracted rings) were determined by log-logit regression. Concentration–response curves were compared by the 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. NO_x formation from oxime derivatives

Oxime derivatives gave rise to NO_2^- or NO_3^- (NO_x) only to negligible extent in Krebs solution (less than 0.1/ml of the initial concentration within 1 hr at 37°). For formamidoxime, acetaldoxime, acetone oxime, acetohydroxamic acid and formaldoxime, the concentration used in the NO_x assay (20 mM) was 200 times higher than the one eliciting 70–95% relaxation (see Fig. 2). Hydroxyguanidine and hydroxyurea (i.e. almost inactive compounds for relaxation, see Fig. 2) produced the largest amount of NO_3^-. Oxime derivatives were compared to DEA-NO and GTN. The two latter compounds were used at a concentration (20 μM) which was also 200 times higher than the ones eliciting at least 85% relaxation (see Fig. 5). As expected, GTN produced very small amount of NO_x in Krebs solution, while DEA-NO was almost completely converted into NO_x (90% being NO_2^-).

3.2. Effect of oxime derivatives on isolated rat aorta: role of the different vascular tunicae

In intact rat aortic rings (i.e. in rings with endothelial, medial and adventitial layers) precontracted with PHE,

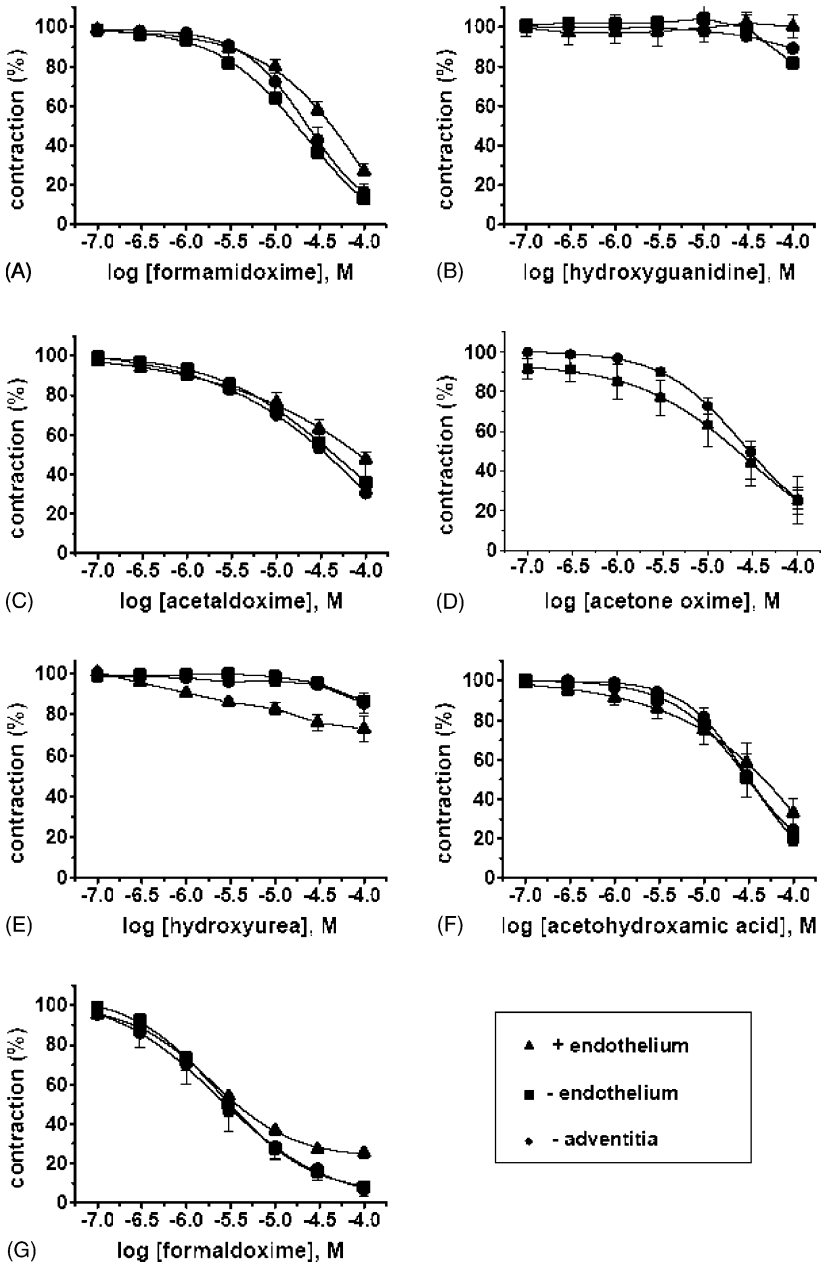

Fig. 2. Effect of formamidoxime (A), hydroxyguanidine (B), acetaldoxime (C), acetone oxime (D), hydroxyurea (E), acetohydroxamic acid (F) and formaldoxime (G) in rat aortic rings with endothelium and adventitia (black triangles), in rings without endothelium and with adventitia (black squares) and in rings without endothelium and adventitia (black circles). Results are mean ± SE mean of at least six experiments.

formamidoxime (Fig. 2A), acetaldoxime (Fig. 2C), acetone oxime (Fig. 2D), acetohydroxamic acid (Fig. 2F) and formaldoxime (Fig. 2G) elicited a concentration-dependent relaxant effect. By contrast, hydroxyguanidine (Fig. 2B) and hydroxyurea (Fig. 2E) were almost ineffective. The effect of all these compounds was not modified after removal of the endothelium or after removal of the endothelium and adventitia (Figs. 2A–G). The most potent relaxant compound was formaldoxime (EC_{50} value in endothelium-denuded rings of about 3 μM). The EC_{50} values of all the other active compounds were in the range of 20–30 μM.

3.3. Relaxant effect of oxime derivatives: pharmacological characterization

In endothelium-denuded rings, the relaxant effect of formamidoxime (Fig. 3A), acetaldoxime (Fig. 3B), acetone oxime (Fig. 3C), acetohydroxamic acid (Fig. 3D) and formaldoxime (Fig. 3E) was inhibited by PTIO (300 μM, a cell-permeable scavenger of NO), by ODQ (1 μM, an inhibitor of the activation soluble guanylyl-cyclase by NO) and by Rp-8-Br-cGMPS (100 μM, an inhibitor of cyclic GMP-dependent protein kinases). It should be noted that in comparison to ODQ, PTIO and Rp-8Br-cGMPS partially inhibited the effect of formamidoxime (Fig. 3A) and formaldoxime (Fig. 3E). By themselves, these inhibitors did not affect PHE-induced tone (not shown).

The relaxant effect of formamidoxime (Fig. 4A), acetaldoxime (Fig. 4B), acetone oxime (Fig. 4C), acetohydroxamic acid (Fig. 4D) and formaldoxime (Fig. 4E) was not inhibited by L-NAME (300 μM, an inhibitor of NOS), proadifen (30 μM, an inhibitor of cytochromes P450) or tranylcypromine (30 μM, an inhibitor of monoamine oxidases and prostacyclin synthase). Like L-NAME, N^{ω}-nitro-L-arginine (L-NA, 300 μM) failed to inhibit the relaxant effect of formaldoxime (not shown). An enhancement of the relaxant effect of formaldoxime was observed in the presence of proadifen and tranylcypromine (Fig. 4E). 17-ODYA (30 μM, a suicide inhibitor of cytochromes P450) did not modify the relaxant effect of oxime derivatives, except the one of acetaldoxime

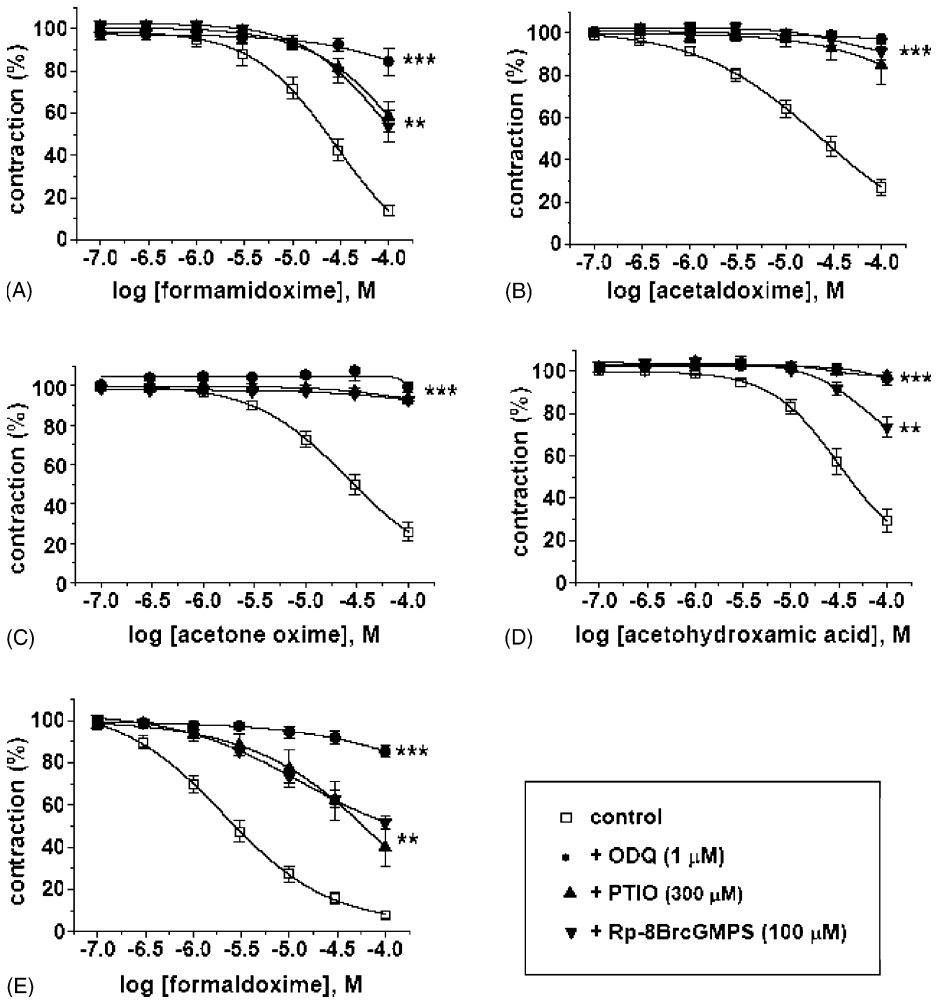

Fig. 3. Effect of PTIO (300 μM, black triangles), ODQ (1 μM, black circles) and Rp-8-Br-cGMPS (100 μM, black squares) on the relaxant effect of formamidoxime (A), acetaldoxime (B), acetone oxime (C), acetohydroxamic acid (D) and formaldoxime (E) in endothelium-denuded rat aortic rings. Results are mean ± SE mean of at least six experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, in comparison to controls.

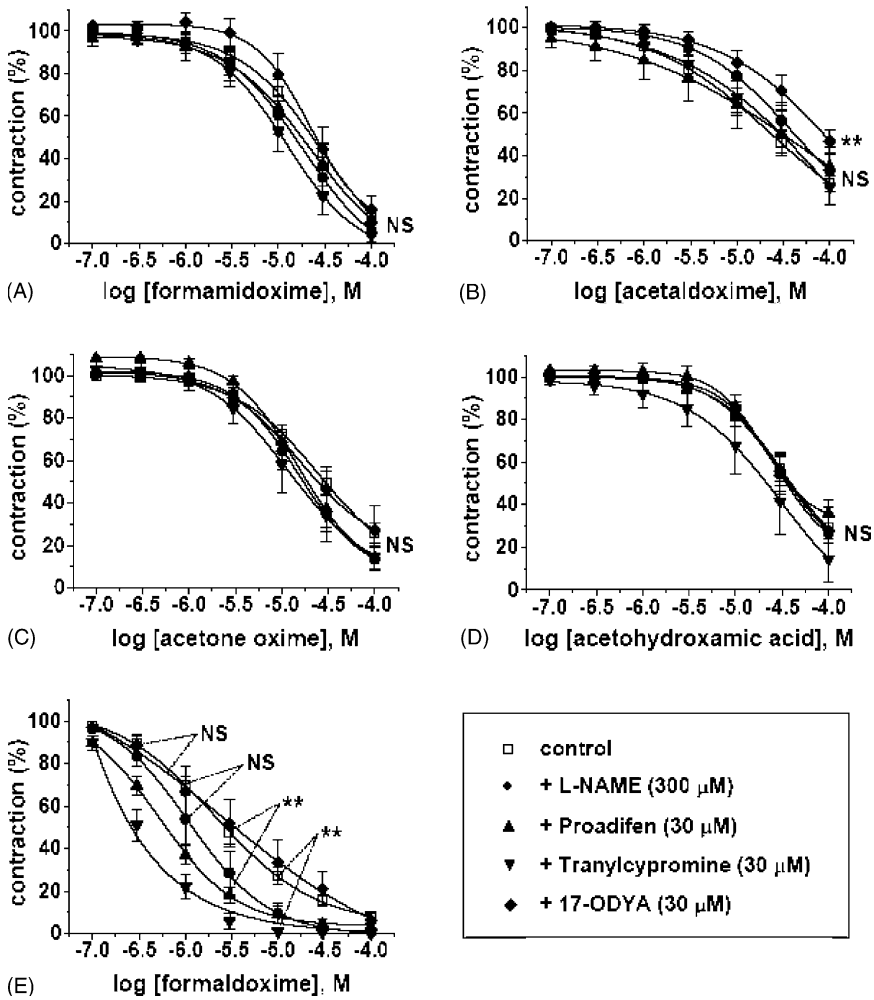

Fig. 4. Effect of L-NAME (300 μM, black circles), proadifen (30 μM, black triangles), 17-ODYA (30 μM, black diamonds) and tranylcypromine (30 μM, black squares) on the relaxant effect of formamidoxime (A), acetaldoxime (B), acetone oxime (C), acetohydroxamic acid (D) and formaldoxime (E) in endothelium-denuded rat aortic rings. Results are mean ± SE mean of at least five experiments. NS not significant; $^{**}P < 0.01$, in comparison to controls.

which was slightly reduced (Fig. 4B). By themselves, none of the inhibitors used influenced PHE-induced tone (not shown).

7-ER (2 μM) almost completely blunted the relaxant effect of formamidoxime (Fig. 5A), acetaldoxime (Fig. 5B), acetone oxime (Fig. 5C), acetohydroxamic acid (Fig. 5D) and formaldoxime (Fig. 5E). By contrast, 7-ER did not modify the relaxation induced by DEA-NO (Fig. 5F) or GTN (Fig. 5G). PHE-induced tone was not affected by 7-ER (not shown).

Pre-exposure of endothelium-denuded rings to GTN (100 μM GTN for 1 hr) resulted in a significant decrease of the relaxant effect of subsequent addition of GTN (Fig. 6A). By contrast in these rings, the relaxant effect of DEA-NO was not altered (Fig. 6B). In rings tolerant to GTN, the relaxant effect of formaldoxime was not impaired in comparison to controls (Fig. 6C). Pre-exposure to formaldoxime (1 mM for 1 hr) did not induce a modification of the relaxant effect of subsequent addition of formaldoxime (Fig. 6D).

3.4. NO formation from oxime derivatives in rat aorta and HASMC

EPR spectroscopy with [Fe(II)(DETC)$_2$] as spin trap was applied to investigate NO formation. Formamidoxime, acetaldoxime, acetone oxime, acetohydroxamic acid and formaldoxime induced a concentration-dependent elevation of NO levels in endothelium-denuded aortic rings, formaldoxime being the most potent compound (Fig. 7A). 7-ER (2 μM) tended to diminish the elevation of NO produced by formaldoxime (1 mM). Increasing the concentration of 7-ER to 10 μM completely abolished formaldoxime-induced NO elevation (Fig. 7B).

Formaldoxime also increased NO content in HASMC (Fig. 7C). In endothelium-denuded rat aortic rings, the elevation of NO content evoked by 1 mM formaldoxime was comparable in control rings and in GTN-tolerant ones (i.e. those previously exposed to 100 μM GTN for 1 hr and washed out, Fig. 7D). In GTN-tolerant rings as in control

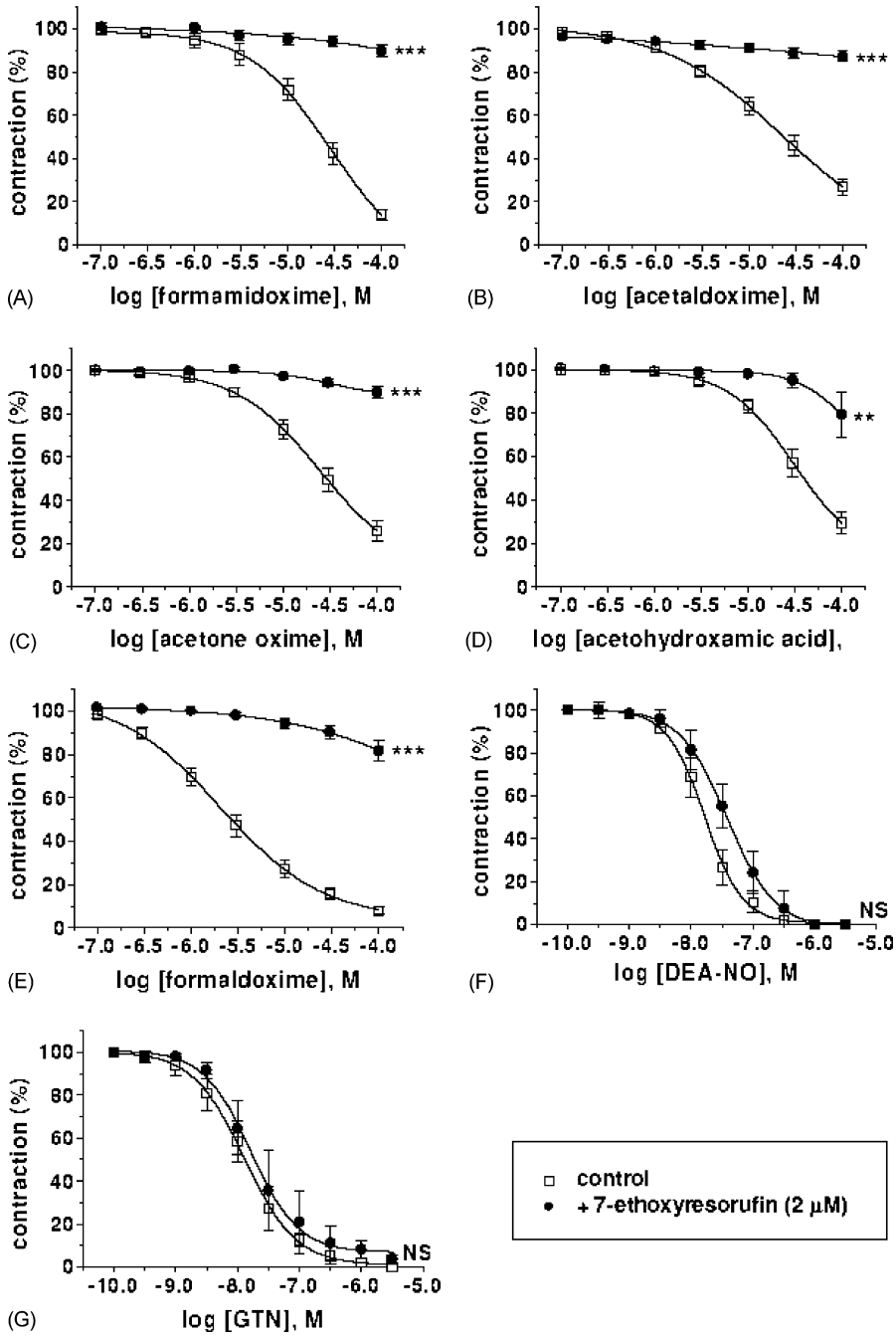

Fig. 5. Effect of 7-ER (2 μM, black circles) on the relaxant effect of formamidoxime (A), acetaldoxime (B), acetone oxime (C), acetohydroxamic acid (D) and formaldoxime (E), DEA-NO (F) and GTN (G) in endothelium-denuded rat aortic rings. Results are mean ± SE mean of at least five experiments. NS not significant; $^{**}P < 0.01$; $^{***}P < 0.001$, in comparison to controls.

ones, 7-ER (10 μM) decreased formaldoxime-induced NO elevation (Fig. 7D).

4. Discussion

The present study shows that in the isolated rat aorta, some oxime derivatives increased NO levels and induced endothelium- and adventitia-independent relaxant effect. Vasorelaxation was decreased by scavengers of NO, inhibitors of soluble guanylyl-cyclase or cyclic GMP-dependent protein kinases, but not by inhibitors of NOS or monoamine oxidases. Vasorelaxation and NO elevation induced by oximes were both blunted by 7-ER, and were not impaired in GTN-tolerant rings.

Among the compounds studied here, hydroxyguanidine was ineffective for vasorelaxation in isolated rat aorta. This is consistent with previous studies performed in rabbit aortic strips [34]. The present study shows that replacement of one amino group of hydroxyguanidine by a hydrogen atom

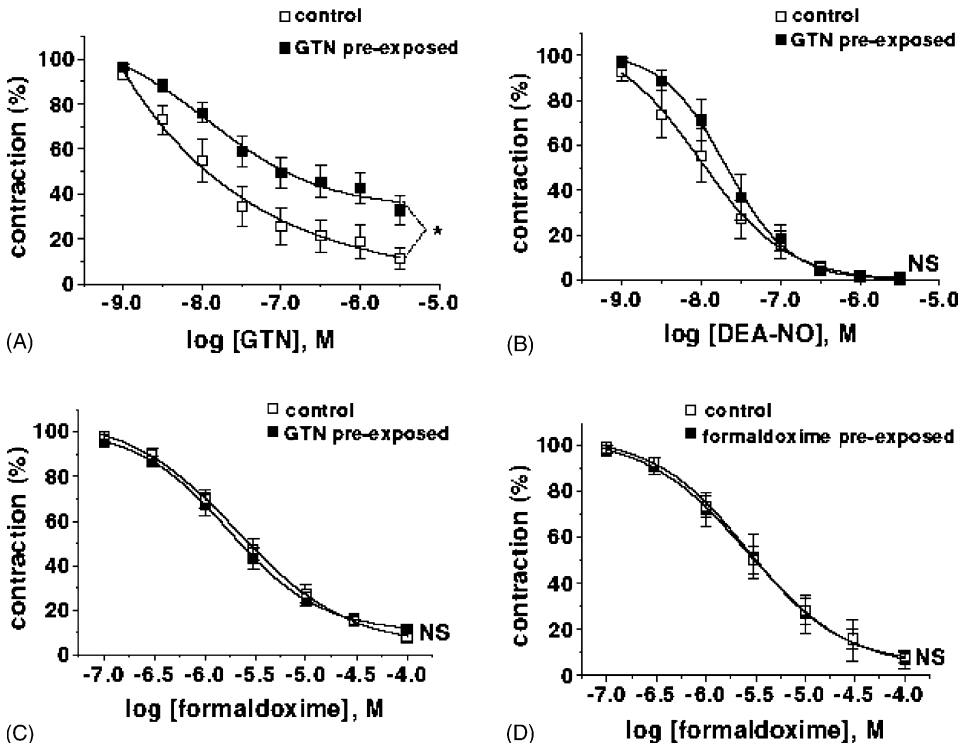

Fig. 6. Effect of GTN (A), DEA-NO (B) and formaldoxime (C) in endothelium-denuded rat aortic rings previously exposed for 1 hr to GTN (100 μM, black squares) or solvent (open squares). (D) Effect of formaldoxime in endothelium-denuded rat aortic rings previously exposed for 1 hr to formaldoxime (1 mM, black squares) or solvent (open squares). Results are mean ± SE mean of at least four experiments. NS not significant; $^{*}P < 0.05$, in comparison to controls.

(formamidoxime) or replacement of its two amino groups by two methyl groups (acetone oxime) markedly increased the vasorelaxant activity. Replacement of the amino group of formamidoxime by a methyl group (acetaldoxime) has no major influence on relaxant activity. The ketoximes derivatives, acetaldoxime (with one methyl group) and acetone oxime (two methyl groups) displayed similar relaxant activity. Acetohydroxamic acid (a hydroxyamide derivative) also exerted vasorelaxant effect in rat aorta, with a potency similar to the one of the above-mentioned amidoxime and ketoxime derivatives. Replacement of the methyl group of acetohydroxamic acid by an amino group (hydroxyurea) abolished relaxant activity. Interestingly, recent reports suggest that the beneficial effects of hydroxyurea in sickle cell disease patients might be partly due to the formation of NO [35,36]. The sites and mechanisms of NO formation from hydroxyurea remain unidentified. The present data suggest that vascular tissues play a minor role in metabolism of hydroxyurea into NO. The weak relaxant effect of hydroxyurea which was observed here in aortic rings with endothelium might be due to production of NO from hydroxyurea by NOS-3 or by other endothelial metabolic pathways. This deserves future investigations.

In the present study, the involvement of the NO-cyclic GMP pathway in the relaxant properties of oxime derivatives is supported by several experimental data. Relaxations were inhibited by PTIO (a cell-permeable NO scavenger), by ODQ (an inhibitor of the activation of soluble guanylyl-cyclase by NO) and by Rp-8-Br-cGMPS (an inhibitor of cyclic GMP-dependent protein kinases). However, it is not excluded that NO-cyclic GMP-independent pathway(s) may contribute to the relaxant effect of some oxime derivatives. Indeed, PTIO and Rp-8Br-cGMPS only partially inhibited the effect of formamidoxime or formaldoxime. Moreover, even though ODQ completely inhibited the relaxant effect of oximes, this compound displays additional properties than inhibition of soluble guanylyl-cyclase in isolated blood vessels [26,37]. Nevertheless, the present study demonstrates using NO spin trapping that active compounds for relaxation increased NO content in aortic tissue. It should be noted that formaldoxime was the most potent compound for both relaxation and NO elevation in aortic tissue. The inhibition by 7-ER of both oxime-induced relaxation and NO elevation provides additional evidence for the implication of NO in the relaxant effect of these compounds. Probably because of the limited sensitivity of the EPR method of NO detection, relatively high concentrations of oxime derivatives had to be used to demonstrate an increase in NO content. Since in the absence of aortic tissue, the active compounds gave rise to very few amount of NO_x in physiological solution, NO elevation in aorta unlikely resulted from spontaneous formation of NO in the bathing solution, but rather from metabolization of the compounds into NO within tissue. Neither endothelial cells nor adventitial cells (which are another source of vasoactive NO in rat aortic rings [25,38]) appeared necessary for NO formation from oxime derivatives, since the relaxant

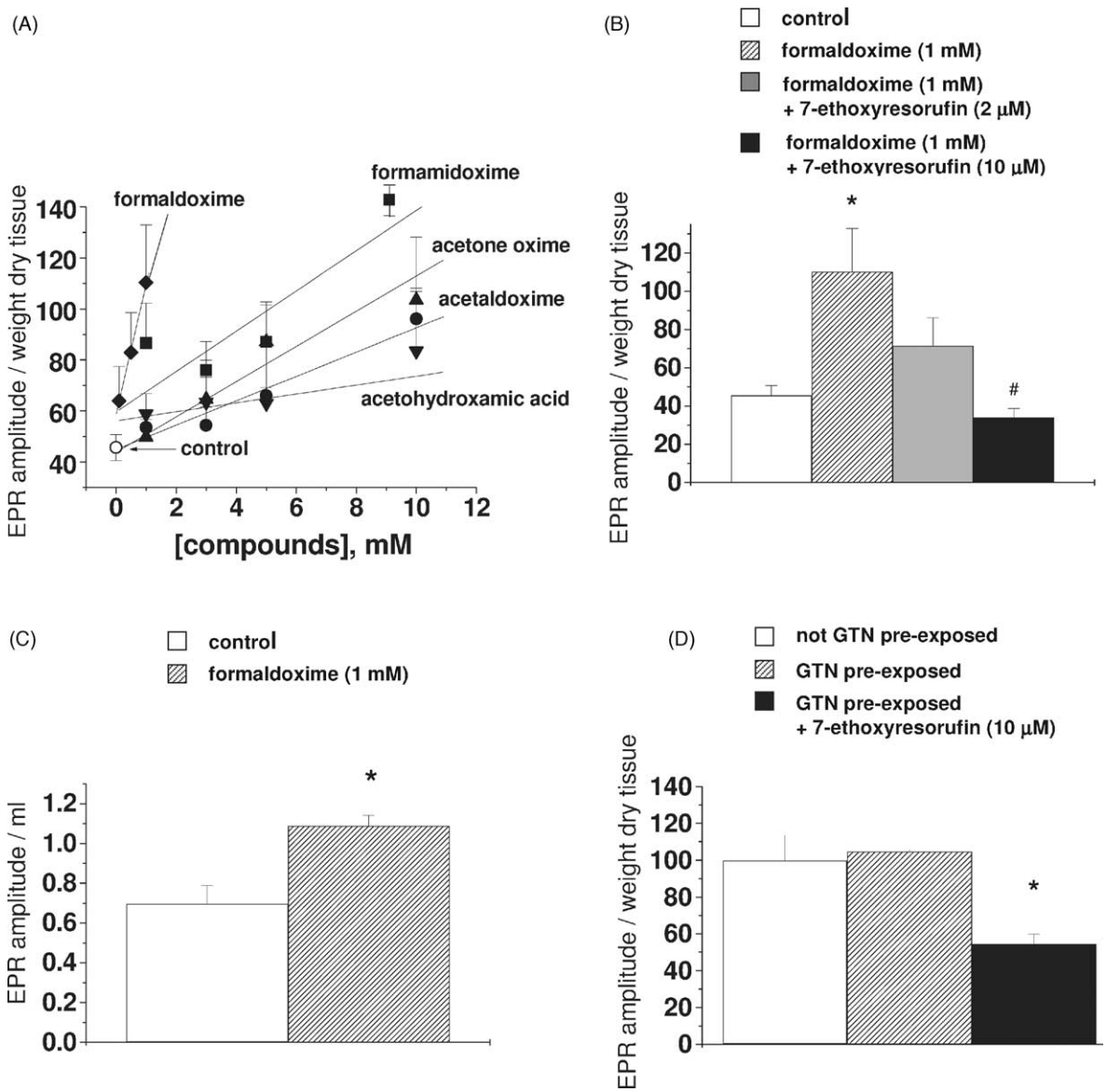

Fig. 7. NO content in (A) endothelium-denuded rat aortic rings exposed to increasing concentrations of formamidoxime, acetaldoxime, acetone oxime, acetohydroxamic acid and formaldoxime; control corresponds to non-stimulated rings (i.e. rings not exposed to oximes derivatives) and (B) endothelium-denuded rat aortic rings exposed or not to formaldoxime (1 mM) in the absence (hatched bars) or in the presence of 2 μM 7-ER (grey bars) or 10 μM 7-ER (black bars); $^{*}P < 0.05$, in comparison to control; $^{\#}P < 0.05$, in comparison to formaldoxime alone; (C) HASMC exposed or not to formaldoxime (1 mM); $^{*}P < 0.05$, in comparison to control; (D) control (white bars) or GTN-pretreated (100 μM for 1 hr, hatched bars) endothelium-denuded rat aortic rings exposed to formaldoxime (1 mM) in the absence or in the presence of 7-ER (10 μM, black bars); $^{*}P < 0.05$, in comparison to GTN-exposed aorta. Results are mean ± SE mean of at least three experiments.

effect was similar in rings with or without endothelium or with or without adventitia. This suggests that metabolization of oximes into vasorelaxing NO occurred within smooth muscle cells. However, oximes can be transformed into NO in endothelial and/or adventitial cells as well. This requires further investigations. The ability of aortic smooth muscle cells to produce NO from oximes is demonstrated in the present study by EPR and NO spin trapping.

The metabolic pathways responsible for NO production from oxime derivatives were investigated in the present study using various inhibitors. The absence of inhibitory effects of L-NA or L-NAME on relaxation in endothelium-denuded rings does not support a role of extra-endothelial NOS in NO production. Moreover, as previously shown for aromatic substituted amidoximes and ketoximes, the structural determinants for endothelium-independent vasorelaxant effect and for NO production by NOS are quite different [18]. The lack of inhibitory effects of tranylcypromine argues not only against a role of monoamine oxidases in oxime metabolization into NO, but also against a contribution of prostacyclin in vasorelaxation [39–41]. Besides NOS, cytochromes P450 have been also implicated in nitrogen oxides production from various compounds bearing a C=NOH group [3,4,6–9]. In the present study, the non-selective cytochromes P450 inhibitor proadifen did not diminish oxime-induced relaxation. Simi-

larly, 17-ODYA, a suicide inhibitor of cytochromes P450, did not modify the relaxant effect of oxime derivatives, except the one of acetaldoxime which was slightly diminished. These data suggest that in rat aorta, cytochromes P450 do not play a major role in NO production from oxime derivatives. However, the possible role of a proadifen and 17-ODYA-insensitive unknown isoform of cytochromes P450 cannot be excluded. The mechanisms responsible for the potentiating effect of proadifen and tranylcypromine on formaldoxime-induced vasorelaxation remain to be determined. Because potentiation was also observed with Tiron (a scavenger of superoxide anions) or with Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP, a cell-permeant superoxide dismutase/catalase mimetic and peroxynitrite scavenger [42–44]) (not shown), it is possible that cytochromes P450 or monoamine oxidase-derived superoxide inhibited the effect of formaldoxime. In addition, the lack of inhibitory effect of MnTMPyP on relaxation did not support a role of hydrogen peroxide and of peroxynitrite as mediator of the relaxant effect of formaldoxime.

An almost complete inhibition of the relaxant effect of oximes (but not of DEA-NO) was obtained here using 7-ER. This compound also completely blunted the increase of NO content induced by formaldoxime in aortic rings. As mentioned above, relatively high concentrations of oximes were used to demonstrate EPR-detectable NO elevation in aortic rings. Thus, a competition phenomenon might explain why relatively high concentration of 7-ER was also necessary to demonstrate significant inhibition of oxime-induced NO elevation. These data indicate that 7-ER inhibited the relaxant effect of oximes by interfering with the metabolic pathways responsible for NO formation. Similar data were obtained with formamidoxime in rat tracheal smooth muscle [8]. 7-ER is a competitive inhibitor of cytochrome P4501A_1 [19] and it also interacts with NADPH-dependent reductases [20,21]. Because identified cytochromes P450 do not seem to play a major role in NO production from oxime derivatives in rat aorta (as discussed above), it is suggested that oxime metabolism into NO is mediated by 7-ER-sensitive NADPH-reductases. A similar pathway is probably involved in NO formation from L-NOHA and 4-chlorobenzamidoxime (an aromatic substituted amidoximes) in rat aorta [18].

Several enzymatic pathways have been proposed in vascular bioactivation of organic nitrates, including glutathione-*S*-transferases, cytochromes P450 [45,46] and mitochondrial aldehyde dehydrogenase (mtALDH) [17]. Whatever the nature of these pathways, the present study suggests that the ones responsible for NO production from oxime derivatives are distinct from those of GTN. Indeed, while it produced almost complete inhibition of the relaxant effect of oximes, 7-ER did not affect the one of GTN. This contrasts with data from the literature showing that 7-ER inhibits the relaxant effect of GTN (and also the one of authentic NO, sodium nitroprusside and acetylcholine) in rat aorta [28]. This discrepancy cannot be attributed to the use of different drug concentration or incubation time, or to presence/absence of endothelium. However, it is known that there exist differences among animal species and vessel types in the sensitivity of GTN-induced relaxation or biotransformation to 7-ER or other inhibitors [28,45]. In the study of Li and Rand [28], experiments were conducted in rat aorta isolated from Sprague–Dawley rats, whereas in the present study, aorta from Wistar rats was used. Thus, the differential sensitivity of GTN-induced relaxation to 7-ER could be due to the use of different strains of rats. To further distinguish between GTN and oximes metabolizing pathways, GTN-tolerant rat aortic rings were also used in the present study. It is known that prolonged exposure of isolated vessels to relatively high concentration of GTN induces a tolerance to the effect of subsequent addition of GTN, which is attributed to an impairment of GTN biotransformation [47–50]. Recent data concerning the mechanism of GTN bioactivation indicate that attenuated biotransformation of GTN by mtALDH underlies the induction of nitrate tolerance in isolated aorta [17]. In accordance with an impaired biotransformation of GTN, it is shown here that aortic rings pre-exposed to GTN exhibited a decrease in the relaxant effect of subsequent addition of GTN, but not in the one of DEA-NO. In GTN-tolerant rings, formaldoxime-induced relaxation and NO elevation were not different to those obtained in control rings, and 7-ER still blunted NO elevation. The absence of cross tolerance between GTN and oximes derivatives further supports the idea that the metabolic pathways of these two categories of compounds into NO are distinct. Interestingly, in rings previously exposed to large concentration of formaldoxime, the effect of subsequent addition of the compound was not affected. This suggests that, in contrast to the metabolic pathways of GTN, those of oxime derivatives are not subjected to tolerance.

In conclusion, this study shows that some oxime derivatives increased NO levels in rat aorta as well as in HASMC, and exerted vasorelaxant effect. Relaxation did not result from spontaneous NO production in the bathing solution, but rather from NO formation in smooth muscle and subsequent activation of soluble guanylylcyclase and cyclic GMP-dependent protein kinases. The pathways responsible for NO production from these compounds were likely distinct from NOS, monoamine oxidases and from those involved in GTN biotransformation. Oxime derivatives could thus be useful for NO delivery in arteries in which endothelial NOS activity is impaired.

Acknowledgments

The authors thank Min-Ho Oak, Jasser El'Bedoui and Pr Valérie B. Schini-Kerth (UMR 7034) for preparation of HASMC. This work was partially supported by grants from Fondation de France and Barrande (00967ZD). K.C. and

P.B. were recipients of fellowships of the French Embassy in Prague. I.L. (Visitor Scientist from Institute of Chemical Physics, Russian Academy of Science, Moscow) was recipient of a fellowship from ARC and Collège Doctoral Européen.

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