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HYDROXYLAMINE IS A VASORELAXANT AND A POSSIBLE INTERMEDIATE IN THE OXIDATIVE CONVERSION OF L-ARGININE TO NITRIC OXIDE

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Our objective was to determine whether hydroxylamine is a possible intermediate in the oxidative conversion of L-arginine to nitric oxide. Vasorelaxation by hydroxylamine is known to be mediated by nitric oxide. The vasorelaxant properties of hydroxylamine were examined using rat aortic rings and an isolated rat lung perfusion model. Hydroxylamine and acetylcholine were equally effective in relaxing norepinephrine-contracted intact aortic rings, whereas only hydroxylamine relaxed aortic rings with endothelium removed. This endothelium-independent vasorelaxation by hydroxylamine indicated that the hydroxylamine-converting enzyme is not localized solely within endothelial cells. Catalase, an enzyme known to oxidize hydroxylamine to nitric oxide, was present in homogenates of intact and endothelium-denuded rings. Cyanamide, another catalase substrate and a known precursor of nitroxyl (HNO), was not a vasorelaxant of aortic rings or of isolated, hypoxia-constricted lungs . These results suggest that free nitroxyl is not an intermediate in the oxidation of hydroxylamine to nitric oxide. An overall pathway for the oxidative conversion of L-arginine through an hydroxylamine intermediate to nitric oxide is proposed.

Hydroxylamine can cause the relaxation of rabbit and rat aortic strips (1,2) and, like nitric oxide (NO), is an activator of guanylate cyclase in various cell-free and intact cell preparations (3-6). Hydroxylamine is also a known precursor of nitric oxide in biological systems (7). The vasorelaxant properties of hydroxylamine are attributed to hydroxylamine-derived nitric oxide rather than to a direct action by hydroxylamine (8,9).

Endothelial cells and immunostimulated macrophages can both convert L-arginine to nitric oxide (and L-citrulline) by apparently similar pathways (10). ^{15}N-Labeling studies have shown that the nitrogen of nitric oxide is derived exclusively from one of the two equivalent guanido nitrogens of L-arginine (10-12). Marletta *et al.* (13) have proposed a complex metabolic pathway for the conversion of L-arginine to nitric oxide as follows: (a) a two-electron oxidation of one of the guanido nitrogens generating N^G-hydroxy-L-arginine by a monooxygenase-type reaction; (b) a two-electron oxidation of N^G-hydroxy-L-arginine to a nitrosoamidine-like intermediate; (c) a one-electron oxidation of this latter intermediate (via extraction of a hydrogen atom) followed by the

Abbreviations: AII, Angiotensin II; EC_{50}, concentration of drug producing 50 percent maximal response; GTN, glyceryl trinitrate; HA, hydroxylamine; NE, norepinephrine; PAP, pulmonary arterial pressure.

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elimination of nitric oxide yielding the diimide of ornithine; and (d) the hydrolysis of this diimide to form L-citrulline.

In this report, we confirm the vasorelaxant properties of hydroxylamine using rat aortic rings and a whole lung rat perfusion model (14) and propose a less complex, alternative pathway for the formation of L-arginine-derived nitric oxide. The proposed pathway is based on the reaction intermediates, N^G-hydroxy-L-arginine (as above) and hydroxylamine. Indirect evidence is also provided which suggests that the conversion of hydroxylamine to nitric oxide does not proceed though a free nitroxyl (HNO) intermediate.

MATERIALS AND METHODS

Materials. Hydroxylamine, cyanamide, angiotensin II and meclofenamate were obtained from Sigma Chemical (St. Louis, MO). All other chemicals used were of reagent quality.

Isolated thoracic aortic rings. Pathogen-free, male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The thoracic aorta was dissected free, excised and placed into cold modified Kreb's-Ringer bicarbonate solution containing the following: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 26 μM calcium disodium edetate and 11.1 mM glucose. The vessels were cut into rings, 6 mm in length. Where indicated, the rings were stripped of endothelial cells by gentle rubbing of the intimal surface with a small forceps (15). The rings were suspended between two stirrups in vessels containing 25 ml of the above modified Kreb's-Ringer solution at 37° and aerated with 95 % O_2/5 % CO_2. The rings were attached to force transducers and the changes in isometric force were recorded (16).

Isolated perfused rat lungs. Animals were anesthetized as above and, after tracheostomy, were mechanically ventilated. The pulmonary artery was cannulated with a double lumen catheter for measurement of perfusion pressure and delivery of perfusate to the lungs. The left atrium was cannulated with a PE 320 cannula. The perfusate (Krebs solution containing 4.0 % albumin and 5 μg/ml of meclofenamate) was recirculated through the lungs at 0.04 ml/min/g rat body weight and was maintained at 37° using a 40 ml heated reservoir. The lungs were ventilated with humidified gases (normoxia: 20 % O_2, 5 % CO_2 and 75 % N_2). The heart and lungs were dissected free, en bloc, and suspended over a water bath in a humidified chamber.

The protocol for this study involved two cycles, each of which lasted 24 min. Pulmonary arterial pressure (PAP) was constantly monitored (Hewlett-Packard recorder) using a small catheter placed inside the pulmonary arterial cannula. During each cycle, the lungs were ventilated with normoxic gas for 10 min and then given a bolus of angiotensin II (0.15 μg) via the pulmonary artery inflow catheter. The peak rise in PAP was allowed to return to baseline over 8 min. During the final six min of the cycle, the lungs were ventilated with hypoxic gas (2.5 % O_2, 5.0 % CO_2 and 92.5 % N_2). When the peak PAP was observed (3 min into hypoxia), the isotonic saline vehicle (200 μl), 4.0 μmol hydroxylamine or 4.0 μmol cyanamide was given via the pulmonary arterial catheter. Perfusate gases were measured before and after administration of angiotensin II and during hypoxia.

These studies were performed in adherence with guidelines established in the *Guide for the Care and Use of Laboratory Animals* published by the U.S. Department of Health and Human Resources (NIH Publication 85-23, revised 1985). Animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the research protocol was approved by the Animal Study Subcommittee of the Minneapolis VA Medical Center.

Catalase measurements. Rat aortic rings with and without endothelium were homogenized in 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 percent Triton X-100 (30 μl buffer per mg wet weight tissue) using a glass/glass conical homogenizer. The homogenate was centrifuged for 10 min at low speed (4° C) to remove tissue debris. The catalase activity in the tissue supernatant was determined using a Yellow Springs Oxygen Monitor equipped with a Clark-style oxygen electrode as previously described (17). Protein was determined using bicinchoninic acid reagent with bovine serum albumin as the standard (18). Catalase activity is expressed as nmol oxygen formed per min per μg protein.

RESULTS

Intact and endothelium-denuded thoracic aortic rings prepared from pathogen-free Sprague-Dawley rats were contracted with norepinephrine (NE; 3 x 10^{-7} M). Increasing concentrations of hydroxylamine or acetylcholine (10^{-9} to 10^{-4} M) were added to the contracted vessels. Acetylcholine and hydroxylamine were about equally effective in causing relaxation of the intact rings (Fig. 1), whereas hydroxylamine, but not acetylcholine, relaxed endothelium-denuded rings (Fig. 2). The EC_{50} values for the concentration response curves are given in Table I.

The endothelium-independent relaxation observed with hydroxylamine suggested that catalase or another oxidative enzyme capable of converting hydroxylamine to nitric oxide is not localized exclusively within the endothelium of the aortic rings. Indeed, the catalatic activity of catalase in the supernatant of ring homogenates prepared from aortic rings with and without endothelium, was found to be 2.92±0.08 and 2.82±0.21 nmol oxygen formed per min per μg protein, respectively. Therefore, the conversion of hydroxylamine to nitric oxide by catalase could account for the observed hydroxylamine-mediated relaxation of the aortic rings.

The oxidation of hydroxylamine to nitric oxide by catalase involves a three-electron oxidation. If this oxidation were to proceed through a two-electron oxidation followed by a one-electron oxidation, nitroxyl (HNO) would be the expected intermediate. To test this possibility, cyanamide, a catalase substrate that yields free nitroxyl (19), was evaluated using endothelium-intact rings (Fig. 3). Cyanamide caused no relaxation of the norepinephrine-contracted rings

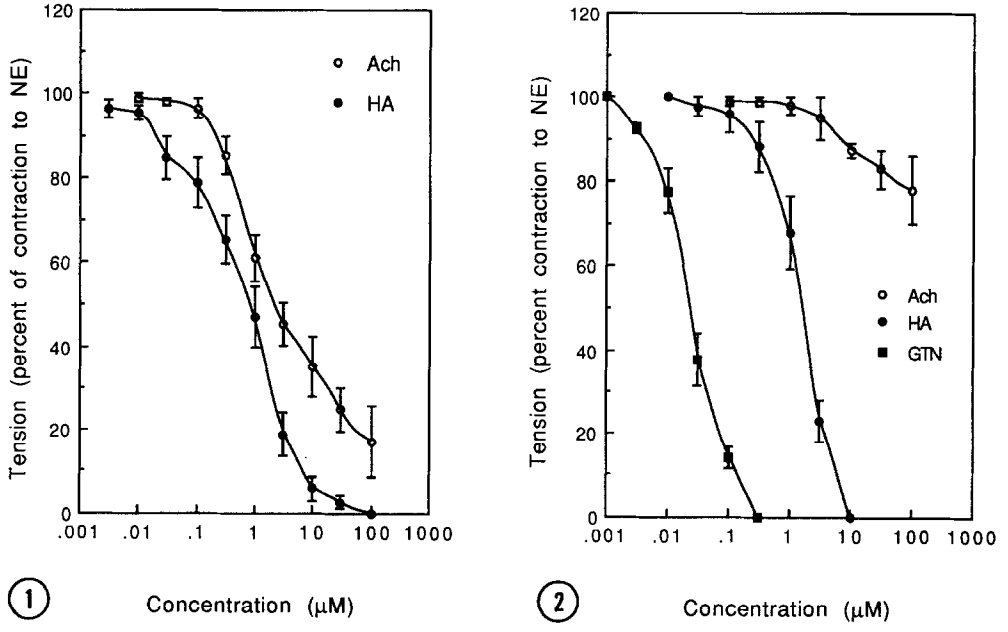

Figure 1. Concentration response curves for the acetylcholine (Ach) and hydroxylamine (HA)-induced relaxation of NE-contracted aortic rings with intact endothelium. The data are presented as means ± S.E.M. of four experiments.

Figure 2. Concentration response curves for the acetylcholine, hydroxylamine and glyceryl trinitrate (GTN)-induced relaxation of NE-contracted, endothelium-denuded aortic rings. The data are given as means ± S.E.M. of four experiments.

Table I

Summary of the EC_{50} Values for the Relaxation of NE-Contracted Aortic Rings by Acetylcholine, Hydroxylamine, Cyanamide and Glyceryl Trinitrate

Relaxant	EC_{50} Value (M)
A. Intact Rings.	
1. Acetylcholine	1.4×10^{-6} *a*
2. Hydroxylamine	6.5×10^{-7}
3. Cyanamide	$> 10^{-4}$
B. Endothelium-denuded Rings.	
1. Acetylcholine	$> 10^{-4}$
2. Hydroxylamine	1.7×10^{-6}
3. Glyceryl trinitrate	2.8×10^{-8}

a Average of the EC_{50} values for the data shown in Figs. 1 and 3.

indicating that neither cyanamide nor cyanamide-derived nitroxyl is vasodilatory and that free nitroxyl is not a precursor of nitric oxide.

The vasodilatory properties of hydroxylamine and cyanamide were also evaluated using perfused lungs preconstricted by ventilation with hypoxia. Hydroxylamine (4.0 μmol) administered at the peak of the hypoxic pressure response resulted in an immediate decrease in PAP (-30±5 percent; -4.7±0.08 mm Hg) (Fig. 4). As observed with the rat aortic rings above,

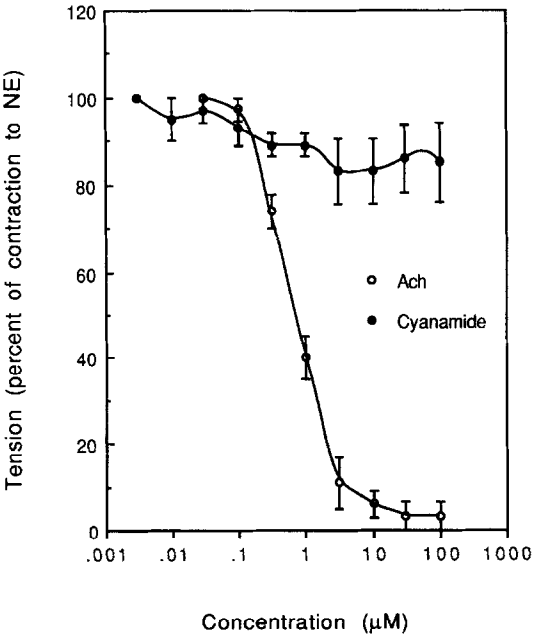

Figure 3. Concentration response curves for the cyanamide and acetylcholine (control) induced relaxation of NE-contracted aortic rings with endothelium intact. The data are presented as means ± S.E.M. of three experiments.

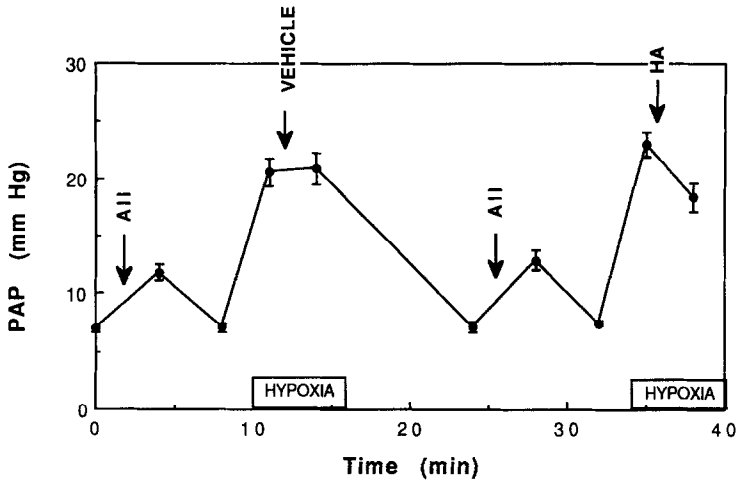

Figure 4. Vasorelaxation of hypoxia-constricted vasculature of the perfused rat lung following a single dose (4.0 μmol) of hydroxylamine. The pulminary arterial pressures (PAP) are expressed as mm of Hg. The data are given as means ± S.E.M. of eight experiments. AII equals angiotensin II.

cyanamide (4.0 μmol; data not shown) did not elicit any detectable vasodilation in the isolated lung model.

DISCUSSION

The dose response relationships for the vasorelaxation of isolated, intact rat aortic rings showed acetylcholine and hydroxylamine to be about equally potent vasorelaxants (Table I) and were consistent with studies of others (1,2). Unlike acetylcholine, hydroxylamine caused vasorelaxation in the absence of endothelial cells (Fig. 2). The observed endothelium-independent relaxation by hydroxylamine indicated that the hydroxylamine-converting enzyme is not localized solely within the endothelial cells of the aortic rings.

Hydroxylamine does not act on guanylate cyclase directly, but rather requires oxidation to nitric oxide to exert its vasorelaxant effect (8,9). Catalase is known to catalyze this reaction (7) and can mediate the hydroxylamine-induced relaxation (8,9). Indeed, homogenates of *intact* and endothelium-denuded rings showed similar levels of catalase activity, which could explain the endothelium-independent vasorelaxant properties of hydroxylamine. However, it is also possible that another oxidative enzyme may be more effective than catalase in catalyzing this conversion *in vivo*.

Hydroxylamine may be an intermediate in the oxidative conversion of L-arginine to nitric oxide. The oxidation of L-arginine to nitric oxide involves a loss of five electrons from one of its guanido nitrogens; *i.e.*, a change in oxidation state from -3 for guanido nitrogen to an oxidation state of +2 for the nitrogen of nitric oxide. N-Hydroxylation of one of the guanido nitrogens yielding N^G-hydroxy-L-arginine, as reported by Marletta *et al.* (10,13), is a two-electron oxidation. The final three-electron oxidation could occur by a single three-electron oxidation or by two (or three) lesser oxidation steps. In Fig. 5, we propose a pathway for the oxidation of L-arginine to nitric oxide, whereby N^G-hydroxy-L-arginine is hydrolyzed to hydroxylamine and

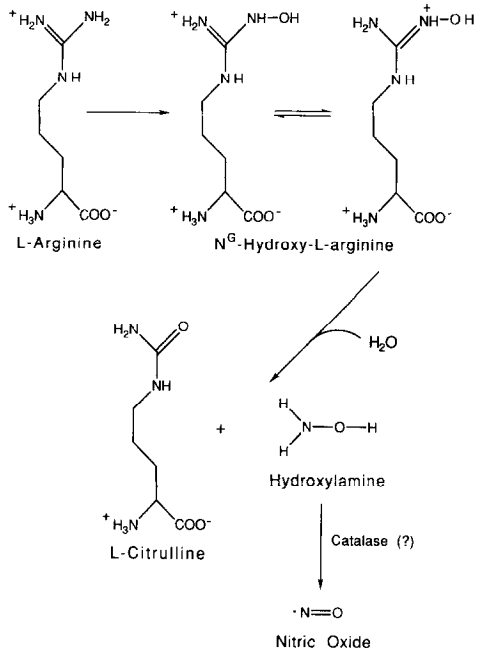

Figure 5. Proposed pathway for the conversion of L-arginine to nitric oxide through a hydroxylamine intermediate.

L-citrulline. The N-hydroxyimine form of N^G-hydroxy-L-arginine would be the expected immediate precusor of hydroxylamine. L-Citrulline has been identified as the co-product of this pathway in endothelial cells and activated macrophages (20, 21).

Hydroxylamine is oxidized in a three-electron oxidation to nitric oxide by catalase. This is achieved through the following series of reactions (eq. 1 to 4):

$$E[Fe^{III}] + H_2O_2 \longrightarrow E[Fe^{V}{=}O] + H_2O \quad \text{(eq. 1)}$$

$$E[Fe^{V}{=}O] + H_2NOH \longrightarrow H_2O + E[Fe^{III}HNO] \longrightarrow E[Fe^{II}NO] + H^+ \quad \text{(eq. 2)}$$

$$E[Fe^{II}NO] \rightleftharpoons E[Fe^{II}] + \cdot NO \quad \text{(eq. 3)}$$

$$E[Fe^{II}] + O_2 \longrightarrow E[Fe^{III}] + O_2^{\cdot -} \quad \text{(eq. 4)}$$

Catalase compound I, $E[Fe^{V}{=}O]$ in eq. 2, oxidizes hydroxylamine to nitric oxide through a ferricatalase-nitroxyl intermediate, $E[Fe^{III}HNO]$. Ferrocatalase, $E[Fe^{II}]$, is converted back to ferricatalase ($E[Fe^{III}]$) by molecular oxygen (22).

Alternatively, the ferricatalase-nitroxyl intermediate could liberate free nitroxyl (and ferricatalase) and free nitroxyl would then be oxidized to nitric oxide by way of a one-electron oxidation. Since cyanamide, a substrate for catalase that is known to yield free nitroxyl, did not elicit a vasodilatory response (Fig. 3), free nitroxyl does not appear to be a precursor of nitric oxide . Therefore, if L-arginine is converted to nitric oxide through an hydroxylamine intermediate, this last step must occur through a three-electron oxidation regardless of whether the oxidation is catalyzed by catalase or another oxidative enzyme.

Direct evidence for hydroxylamine as an intermediate of the proposed pathway is still lacking. This evidence will have to be obtained through the trapping of L-arginine-derived hydroxylamine in ^{15}N-tracer studies.

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