Potentialiation of the vasorelaxant activity of nitric oxide by hydroxyguanidine: implications for the nature of endothelium-derived relaxing factor

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1 We recently demonstrated that N^G-hydroxy-L-arginine (L-HOArg) is a substrate for the constitutive nitric oxide (NO) synthase present in bovine aortic endothelial cells cultured on microcarrier beads (EC). Furthermore, L-HOArg reacts chemically with NO released from these cells to form a potent and more stable vasodilator. This is most likely through a reaction with the hydroxyguanidino group.

2 Here, we studied the interaction of a simpler molecule, hydroxyguanidine (HOG) with NO.

3 HOG (10 µM), like L-HOArg (10 µM) or N^G-hydroxy-D-arginine (D-HOArg, 10 µM), potentiated and stabilized the relaxant activity of authentic NO.

4 When NO was bubbled through the solution of HOG, a new compound was formed. It had similar physicochemical properties to those of the previously described L-HOArg/NO adduct. It was also a potent vasodilator and its action was inhibited by oxyhaemoglobin (10 µM), indicating formation of a NO-containing substance.

5 Moreover, HOG (10 µM) was not a substrate for the constitutive NO synthase present in the microsomal fraction of EC and did not affect the flow-induced or bradykinin-stimulated generation of prostacyclin, as measured by 6-ketoPGF_{1α}.

6 We also studied the effect of HOG on the endothelium-derived relaxing factor (EDRF) released from the column of EC. HOG (10 µM) potentiated and stabilized the relaxations of rabbit aortic strips induced by EDRF released by bradykinin (5–20 pmol) or ADP (5–10 nmol). These relaxations were inhibited by N^G-nitro-L-arginine methyl ester (L-NAME, 10 µM) and L-arginine (L-Arg, 1 mM) reversed the inhibitory effects of L-NAME.

7 HOG (10 µM) augmented the basal (flow-induced) EC-dependent relaxations which were also inhibited by L-NAME (10 µM) and the effects of L-NOArg were reversed by L-Arg (1 mM).

8 Thus, the hydroxyguanidino moiety of L-HOArg is involved in the reaction with NO. Moreover, the comparable reaction of the hydroxyguanidino compounds with NO on the one hand and with flow-induced and agonist-triggered EDRF on the other, strongly supports their common identity.

Keywords: Endothelium; endothelium-derived relaxing factor; nitric oxide; arginine; hydroxyguanidine; N^G-hydroxy-L-arginine; bioassay; h.p.l.c.

Introduction

Nitric oxide (NO) is generated by mammalian cells from one of the N^G-guanidino nitrogens of L-arginine (L-Arg) and this reaction is catalysed by a NADPH-dependent dioxygenase (Kwon *et al.*, 1990). This enzyme, referred to as nitric oxide synthase exists in at least two distinct isoforms. One, a constitutive agonist-triggered and calcium-dependent NO synthase is mainly present in neuronal cells (Bredt & Snyder, 1990) and vascular endothelial cells (EC, Forstermann *et al.*, 1991). The other enzyme, a calcium-independent inducible NO synthase, is found in macrophages (Marletta *et al.*, 1988), hepatocytes (Billiar *et al.*, 1990), endothelial cells (Radomski *et al.*, 1990) and smooth muscle cells (Busse & Mulsch, 1990) after activation by bacterial lipopolysaccharide (LPS) or cytokines.

The formation from L-Arg of NO or a closely related substance by EC is involved in the maintenance of the tone and thromboresistance of the vascular wall (for review see, Vane *et al.*, 1990). In the central and peripheral nervous systems NO functions as a neurotransmitter (for review see, Snyder & Bredt, 1991). In the immune system NO made by

the inducible NO synthase is responsible for the killing of tumour cells and microbial pathogens by activated macrophages (for review see Nathan & Hibbs, 1991).

The exact mechanisms of the conversion of L-arginine to NO by the isoforms of NO synthase are now known. N^G-hydroxylation of L-arginine (L-HOArg) has been proposed as a first step in the biosynthesis of NO (Marletta *et al.*, 1988). This notion was recently confirmed by Stuehr *et al.* (1991) who demonstrated that L-HOArg is, indeed, formed by the inducible NO synthase from macrophages. Moreover, L-HOArg is a substrate for this NO synthase and stable isotope studies revealed that this enzyme oxidizes exclusively the hydroxylated nitrogen of L-HOArg.

Recently we have demonstrated three mechanisms by which L-HOArg induces endothelium-dependent relaxations (Zembowicz *et al.*, 1991). It is a substrate for the constitutive NO synthase present in EC. L-HOArg induces a relatively stable, EC-dependent relaxation which is not affected by inhibitors of NO synthase. It also reacts with NO released from EC to form a potent and more stable vasorelaxant compound. The last of these effects is not specific for the L-isomer of hydroxyarginine. In contrast, L-Arg does not affect the EC-dependent relaxations nor does it react with NO. This suggested to us that the hydroxyguanidino moiety of L-HOArg is responsible for the EC-dependent relaxation or reaction with NO.

Here, we have investigated the effects of hydroxyguanidine (HOG) on the biosynthesis and vasorelaxant activity of

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EDRF released from cultured bovine aortic endothelial cells. We demonstrate that HOG potentiates both the agonist-triggered and flow-induced EC-dependent relaxations and that this is due to the reaction of HOG with NO released from endothelial cells and the formation of a new, more stable compound with similar physicochemical characteristics to those of the L-HOArg/NO adduct. Moreover, these results strongly support the notion that the EDRF released by endothelial cells which mediates the EC-dependent relaxations induced by flow or agonists is, indeed, NO.

Methods

Cell culture

Bovine aortic endothelial cells were harvested and grown on Cytodex-3 microcarrier beads (Pharmacia-LKB Ltd.) in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum (FCS; Gibco) as described previously (De Nucci *et al.*, 1988). LLC-PK₁ pig kidney epithelial cells (ATCC CL 101) were obtained from the European Collection of Animal Cell Cultures and grown on 96-well plates in medium 199 (Flow Laboratories) supplemented with 10% FCS.

Bioassay of endothelium-derived relaxing factor

Endothelium-derived relaxing factor (EDRF) was bioassayed as previously described (Gryglewski *et al.*, 1986). Briefly, male New Zealand White rabbits (2.2–2.8 kg) were anaesthetized with sodium pentobarbitone and their thoracic aorta were rapidly removed. After cleaning of adhering periaortic fat and removal of the endothelium on a glass rod, the aortae were cut spirally into strips (3 × 30 mm). Approximately 9×10^7 endothelial cells cultured on beads (3 ml) were packed into a jacketed chromatography column and perfused at 5 ml min⁻¹ with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs solution containing 5.6 µM indomethacin and in some experiments superoxide dismutase (SOD, 10–30 u ml⁻¹). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.17, CaCl₂ 2.5, NaHCO₃ 2.5, and glucose 5.6. The effluent from the column superfused a cascade of three or four spirally cut strips of rabbit aorta (detector tissues) which were precontracted with 30 nM U46619. Relaxations of the detector tissues were recorded by auxotonic levels attached to Harvard isotonic transducers connected to a Graphtec WR3101 linear-corder. The amplifications of the recorders were adjusted so that standard doses of glyceryl trinitrate (22.5–90 pmol) induced similar recordings of the relaxations of all arterial strips. Bradykinin or ADP were given as bolus injections over the assay tissues (o.t.) or through the column (t.c.). Saturated solutions of NO (1–3 mM) were prepared by bubbling 2.5 ml of NO gas (Merck Ltd.) into 1 ml of helium-deoxygenated water kept in an air tight flask at 0–4°C. An aliquot of this solution (1 µl corresponding to 1–3 nmol) was injected t.c. as a bolus. All hydroxyguanidino-containing compounds were infused at 100 µl ml⁻¹ either o.t. or t.c. In some experiments the column was packed with empty microcarrier beads (3 ml) containing no endothelial cells and experiments were performed as described above.

Preparation of subcellular fractions from cultured endothelial cells

All of the following procedures were carried out at 0–4°C. Approximately 10⁹ bovine aortic endothelial cells cultured on beads were suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA, 5 mM glucose, 1.15% (wt/v) KCl, 0.1 mM DL-dithiothreitol (DTT), 2 mg l⁻¹ leupeptin, 2 mg l⁻¹ pepstatin A, 10 mg l⁻¹ trypsin inhibitor, and 44 mg l⁻¹ phenylmethylsulphonyl fluoride (PMSF). The cell suspension

was bubbled with helium for 15 min, sonicated, and the homogenate centrifuged at 1,000 g for 10 min to remove the empty beads. The 1,000 g-supernatant was centrifuged for 20 min at 10,000 g, the pellet discarded and the 10,000 g-supernatant centrifuged for 30 min at 200,000 g. The 200,000 g-supernatant (cytosol) which contains less than 10% of NO synthase activity (Zembowicz *et al.*, 1991) was discarded and the pellet (microsomes) was resuspended in 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 mM EDTA, 0.1 mM DTT, 2 mg l⁻¹ leupeptin, 2 mg l⁻¹ pepstatin A, 10 mg l⁻¹ trypsin inhibitor, 44 mg l⁻¹ PMSF, and 10% (v/v) glycerol. Protein concentrations were determined by using a modified Lowry-method.

Determination of NO synthase activity

NO synthase activity was measured by monitoring NO-mediated increases in the levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in pig kidney epithelial cell line LLC-PK₁ with some modification of the previously described procedure (Zembowicz *et al.*, 1991). Aliquots of the microsomal fraction (50–100 µg of protein) were diluted in PBS buffer (with Ca²⁺ and Mg²⁺), pH 7.4, containing SOD (100 u ml⁻¹), 100 µM NADPH, 100 µM DTT, 1 µM THB, and 0.1 µM calmodulin and incubated in the presence or absence of HOG, L-Arg, or L-HOArg (all at 100 µM) with the monolayers of LLC-PK₁ cells cultured in 96-well plates (5×10^5 cells/well) which were pretreated for 30 min with 0.5 mM isobutylmethylxanthine. The final volume of incubations was 100 µl. After 10 min, 100 µl of sodium acetate (pH 4.0) was added to each well and plates were rapidly frozen in liquid nitrogen in order to stop the reactions and break the cells. Contents of wells were diluted 50–100 fold with sodium acetate pH 5.6 (assay buffer) and cyclic GMP concentrations were measured by radioimmunoassay after prior acetylation of samples with acetic anhydride (Harper & Brooker, 1975) using a specific antiserum provided by Dr H. Strobach (Institute of Pharmacology, University of Dusseldorf, Germany) and [¹²⁵I]-cyclic GMP obtained from Amersham International (UK). The sensitivity of the assay was approximately 1–2 pmol of NO/ 10 min.

Measurement of 6-keto-PGF_{1α}

The effluent from the column of endothelial cells was collected for 3 min under basal conditions or after a bolus injection of bradykinin (5–20 pmol, t.c.). The levels of 6-keto-PGF_{1α}, a hydrolysis product of prostacyclin, were measured by a radioimmunoassay (Salmon, 1978) using antiserum purchased from Sigma (UK).

Isolation and h.p.l.c. analysis of hydroxyguanidine/nitric oxide adduct

Hydroxyguanidine/nitric oxide adduct was prepared by the reaction of hydroxyguanidine hemisulphate with sodium nitrite in water at pH 3. The precipitated product was dried and stored over calcium chloride at room temperature; 1–2 mg of this powder was reconstituted in distilled water and injected (10 µl) into isocratic high performance liquid chromatography (h.p.l.c.) system consisting of an aminopropyl stationary phase and a mobile phase of 60% acetonitrile and 40 phosphate buffer (10 mM KH₂PO₄, pH 4.3). The fraction corresponding to the HOG/NO adduct gave a single peak of u.v. absorbance at wavelengths of 205 or 320 nm and was collected for the determination of vasorelaxant activity.

Organ bath experiments

Rabbit thoracic aorta was obtained as described above taking care not to damage the endothelium. The aorta was cut into rings 5 mm wide which were suspended between stainless steel wires and mounted in 20 ml organ baths filled with

warmed (37°C) and oxygenated (95% O₂, 5% CO₂) Krebs buffer. Isometric force was measured with Biegestab K30 type 351 transducers (Hugo Sachs Electronic, Germany). A tension of 4 g was applied and rings were equilibrated (60–90 min) with changing the Krebs buffer and adjusting a preload to 4 g every 15 min. After a stable baseline was obtained, rings were contracted with KCl (15 mM) and relaxed with acetylcholine (ACh; 0.3 µM) which was added to the baths in order to verify the functional integrity of the endothelium. Only those rings which relaxed to ACh were used for further studies. After changing the buffer 4 times every 15 min the preparations were contracted with increasing concentrations of phenylephrine (0.01–10 µM) in the absence or presence of HOG (10 µM) which was added to the baths 15 min before addition of phenylephrine. Alternatively, rings were constricted with phenylephrine (0.1 µM) and cumulative concentration-response curves to ACh (0.01–1 µM) were obtained with or without prior preincubation of the tissues in the presence of HOG (10 µM). Contractions were calculated as % of the contraction induced by KCl (15 mM) and relaxations were expressed as % of the tone induced by phenylephrine.

Materials

Indomethacin, adenosine diphosphate (ADP), bradykinin, superoxide dismutase (from bovine erythrocytes; SOD), N^G-nitro-L-arginine methyl ester, were purchased from Sigma Chemical Co. (U.K.), (6R,S)-5,6,7,8-tetrahydro-L-biopterin (THB) from Dr B. Schricks Laboratories (Switzerland), and glyceryl trinitrate (Nitronal) from Lipha Pharmaceuticals Ltd (U.K.). 9,11-Dideoxy-9 α ,11 α -methanoepoxy-prostaglandin F_{2 α} (U46619) was a generous gift from the Upjohn Co. (U.S.A.). Hydroxyguanine sulphate was synthesized by the method of Walker & Walker (1959). L-HOArg was synthesized by the Glaxo Group Research Ltd. (U.K.) and D-HOArg was synthesized by Dr Paul L. Feldman (Medicinal Chemistry, Glaxo Inc. Research Institute, Research Triangle Park, N.C., U.S.A.). Oxyhaemoglobin was prepared by reduction of bovine haemoglobin (obtained from Sigma) with sodium hydrosulphite followed by gel filtration with a prepacked disposable column PD-10 (Pharmacia) equilibrated with Tris-HCl (50 mM) at pH 7.4. All other reagents and solvents were of the highest commercially available quality from either Sigma Chemical Co. (U.K.) or Merck Ltd. (U.K.).

Statistical analysis

All values in the figures and text are expressed as mean $\pm$ s.e.mean of *n* observations. A one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni test or a two-tailed one sample *t* test was used, where appropriate, to assess the statistical significance of results. A *P* value less than 0.05 was considered statistically significant.

Results

Effects of hydroxyguanine on the biological activity of endothelium-derived relaxing factor

HOG infused over the detector tissues (o.t.) at a concentration of 10 µM did not affect the tone of the detector tissues (*n* = 12; Figures 1, 2) and was without effect on the relaxations induced by GTN (*n* = 12; 45–90 pmol; o.t.; Figures 1, 2, 5). However, when HOG (10 µM) was infused through the column of EC (t.c.), in 6 out of 8 experiments there was a relaxation of the detector tissues which was the biggest on the first detector tissue and then smaller but relatively stable down the cascade. As seen in Figure 1 these relaxations were abolished in the presence of L-NAME (10 µM) and the effects of L-NAME were reversed by a concomitant infusion of L-Arg (1 mM).

Figure 1 Hydroxyguanine potentiates the vasorelaxant activity of flow-induced EDRF released from cultured endothelial cells (EC) as detected by the cascade bioassay. For experimental details refer to Methods. In (a) is shown an original trace of three rabbit aortic strips separated from the column of endothelial cells by 1, 4 and 7 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Hydroxyguanine (HOG; 10 µM) infused o.t. did not affect the tone of the detector tissues. When HOG (10 µM) was infused through the column of endothelial cells (t.c.) there was a relaxation of all three detector tissues. These relaxations were abolished in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 10 µM, t.c.) and this abolition was prevented by co-infusion of L-arginine (L-Arg; 1 mM). This trace is representative of a series of experiments (*n* = 3) summarized in graph (b). The relaxations (expressed as % relaxation relative to the response caused by GTN) of the first (solid columns), second (hatched columns) and third (open columns) detector tissues are shown.

HOG (10 µM) infused t.c., potentiated the relaxations induced by EDRF released from endothelial cells by bradykinin (BK, 5–20 pmol; t.c.; *n* = 9) or ADP (5–10 nmol; t.c.; *n* = 6). As depicted in Figure 2, these relaxations were more stable on the cascade than those induced in the absence of HOG. Moreover, they were abolished by L-NAME (10 µM; t.c.) in a manner that was reversible by L-Arg (1 mM; t.c.). Infusion of HOG (10 µM) o.t. did not affect the responses of the first two detector tissues to EDRF. However, on the third detector tissue, a slight potentiation was consistently observed (Figure 2). The effects of HOG were not due to the scavenging by HOG of superoxide anions for similar results were obtained in the presence of SOD (10–30 u ml⁻¹; t.c.; *n* = 5; data not shown).

Metabolism of hydroxyguanine-containing compounds by nitric oxide synthase

We have previously reported (Zembowicz *et al.*, 1991) that the activity of the constitutive NO synthase is largely recovered in the membrane fraction from endothelial cells. The activity of NO synthase was assayed by monitoring NO-mediated increases in the level of cyclic GMP in LLC-PK₁ cells. Incubation of microsomal protein (50–100 µg) with L-Arg (100 µM) or L-HOArg (100 µM) increased the

Figure 2 Hydroxyguanine potentiates the vasorelaxant activity of EDRF released from cultured endothelial cells (EC) by agonists. For experimental details refer to Methods. An original trace is shown of two (out of three) rabbit aortic strips separated from the column of EC by 1 and 4 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. ADP (5 nmol) or bradykinin (BK, 5 pmol) injected through the column of endothelial cells (t.c.), caused the release of EDRF which was detected as the relaxations of the detector tissues which disappeared down the cascade. Hydroxyguanine (HOG; 10 μM) infused o.t. had no effect on the relaxant responses to ADP or BK. When infused t.c., HOG (10 μM) potentiated and stabilized the ADP- or BK-induced relaxations. In the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM, t.c.) these relaxations were blocked and the effect of L-NAME was prevented by co-infusion of L-arginine (L-Arg; 1 mM, t.c.). The results from 4 similar experiments are summarized in (b). The relaxations (expressed as % relaxation relative to the response caused by GTN) of the first (solid columns), second (hatched columns) and third (open columns) detector tissues induced by BK (5–10 pmol; t.c.) are shown. In the absence of HOG, BK induced the relaxations of only the first two tissues. However, in the presence of HOG (10 μM, o.t.) relaxations of the third tissue were also observed. C, control.

**P* < 0.05 when compared to control; †*P* < 0.05 when compared to control.

intracellular concentrations of cyclic GMP in LLC-PK₁ cells by 24 ± 10 pmol mg⁻¹ protein 10 min⁻¹ (*n* = 4 different batches of EC) in the case of L-Arg and by 37 ± 18 pmol mg⁻¹ protein min⁻¹ (*n* = 4) in the case of L-HOArg. HOG (100 μM) had no effect (*n* = 4), indicating that it is not a substrate for NO synthase.

Effect of hydroxyguanine on the generation of prostacyclin by endothelial cells

The effect of HOG on the formation by endothelial cells of prostacyclin was measured by monitoring the concentrations of 6-ketoPGF_{1α}, a product of its hydrolysis. The basal generation of 6-ketoPGF_{1α} was 3.3 ± 0.8 ng 3 min⁻¹ in the absence and 2.9 ± 0.5 ng 3 min⁻¹ in the presence of HOG (10 μM; *n* = 5). Similarly, HOG did not affect the generation of 6-ketoPGF_{1α} by EC stimulated with BK (20 pmol) which was 15 ± 5 in the absence and 11 ± 3 in the presence of HOG (10 μM; *n* = 5).

Potential of the biological activity of authentic nitric oxide by hydroxyguanine

NO (1–3 nmol), injected through the column of empty beads, caused characteristic relaxations of the detector tissues which rapidly diminished down the cascade. HOG (10 μM; t.c.; *n* = 6), L-HOArg (10 μM; t.c.; *n* = 7) or D-HOArg (10 μM; t.c.; *n* = 7) significantly potentiated relaxations induced by NO which in the presence of hydroxyguanine-containing compounds were more stable down the cascade (Figure 3). When HOG (10 μM), L-HOArg (10 μM) or D-HOArg (10 μM) were infused o.t. the relaxant responses to NO (1–3 nmol, t.c.) of only the third, but not the first two, detector tissue were potentiated (*n* = 3; data not shown). These results all strongly suggested that the potentiation of the biological activity of EDRF by HOG is due to a chemical reaction with NO (taking a few seconds) and formation of a novel and more stable vasorelaxant compound.

Figure 3 Hydroxyguanine potentiates the vasorelaxant activity of nitric oxide. For experimental details refer to Methods. In (a) is shown an original trace of three rabbit aortic strips superfused by the effluent from the column containing empty beads and separated from the column by 1, 4 and 7 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Nitric oxide (NO; 1–2 nmol) given through the column (t.c.) caused characteristic relaxations of the detector tissues which disappeared down the cascade. The vasorelaxant activity of NO was significantly increased and stabilized by hydroxyguanine (HOG; 10 μM, t.c.), N^G-hydroxy-L-arginine (L-HOArg; 10 μM, t.c.) or N^G-hydroxy-D-arginine (D-HOArg; 10 μM, t.c.). The results of 4 similar experiments are summarized in the graph (b). The relaxations (expressed as % relaxation relative to the relaxation induced by GTN) of the first, second and third detector tissue are shown in the absence (□; *n* = 18) of hydroxyguanine compounds or in the presence of HOG (10 μM; *n* = 6; ▲), L-HOArg (10 μM; *n* = 7; ●) or D-HOArg (10 μM; *n* = 7; ○). Values represent means ± s.e. mean (vertical bars) of *n* separate measurements. **P* < 0.05 when compared to control.

Figure 4 Hydroxyguanine (HOG) reacts with nitric oxide (NO) to form a novel vasodilator. (a) Typical normal phase h.p.l.c. chromatogram of the HOG/NO adduct (for experimental details refer to Methods). The h.p.l.c. fraction, after injection of 10 μ l of 1 mg ml⁻¹ solution of the HOG/NO adduct, was collected and its vasorelaxant activity assayed using the cascade bioassay. In (b) an original trace of three rabbit aortic strips superfused with Krebs solution separated from each other by 3 s is shown. Glycerol trinitrate (G; 90 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Nitric oxide (N; 1–2 nmol, o.t.) caused the relaxation of the detector tissues which disappeared down the cascade. In contrast, relaxations induced by the h.p.l.c. fraction containing HOG/NO adduct (A; corresponding to approximately 20 ng of compound) were stable. The h.p.l.c. solvent (S; 1 μ l; o.t.) had no vasorelaxant activity. Oxyhaemoglobin (Hb; 10 μ M; o.t.) significantly inhibited vasorelaxant responses to all tested compounds. This figure is representative of 4 experiments using different preparations of the HOG/NO adduct.

Figure 5 Hydroxyguanine (HOG) does not affect the endothelium-dependent relaxations of rabbit aortic rings. Rings were contracted with KCl (15 mM) and the functional integrity of endothelium was confirmed by relaxations to acetylcholine (0.3 μ M). After extensive rinsing, the cumulative concentration-response curves to phenylephrine (0.01–10 μ M; a) were constructed in the presence (●) or in the absence (□) of HOG (10 μ M). Alternatively (b), the rings were preconstricted with phenylephrine (0.1 μ M) and cumulative concentration-response curves to acetylcholine (0.1–1 μ M) were produced in the presence (●) or absence (□) of HOG (10 μ M). In both series of experiments, HOG was added to organ baths 15 min before addition of phenylephrine. Contractions are expressed as % of the contraction induced by KCl and relaxations as % of the phenylephrine-induced tone. Values represent means $\pm$ s.e.mean (vertical bars) of $n = 6$ –12 rings from 6 different animals.

Vasorelaxant properties of hydroxyguanine/NO adduct

HOG (10 mM), when incubated with authentic NO or NO₂⁻ in acid, exhibited u.v. spectrum with a maximum of 320 nm not seen for HOG alone. This HOG/NO adduct was isolated by h.p.l.c. (Figure 4a) and, when injected o.t. in the cascade bioassay, caused a stable relaxation of the detector tissues which was blocked by oxyhaemoglobin (oxy-Hb; 10 μ M; Figure 4b). The h.p.l.c. solvent did not affect the tone of the detector tissues.

Organ bath experiments

Incubation of rabbit aortic rings with HOG (10 μ M) had no effect on the resting tone of the tissues (not shown) and it did not affect the concentration-dependent contractions to phenylephrine (Figure 5a). When rings were preconstricted with phenylephrine (0.1 μ M), ACh produced a concentration-dependent relaxation which was not changed by pretreatment with HOG (10 μ M; 10 min; Figure 5b).

Discussion

Our results demonstrate that HOG potentiates the vasorelaxant activity of EDRF released from bovine aortic endothelial cells by flow as well as by agonists. This effect is due to the chemical reaction of HOG with NO released from endothelial cells and formation of a novel, potent vasodilator.

Both the EC-dependent relaxation induced by HOG as well as relaxations triggered by BK or ADP in the presence or absence of HOG were totally dependent on the activity of the L-Arg/NO pathway, for they were abolished by L-NAME, an inhibitor of L-Arg-dependent NO synthase (Mulsh & Busse, 1990; Rees *et al.*, 1990) and the inhibitory effect of L-NAME was reversed by L-Arg, a substrate for this enzyme (Palmer *et al.*, 1988). The potentiation of the agonist-triggered EC-dependent relaxation cannot be explained by a direct activation by HOG of EC, for HOG had no effect on the generation of prostacyclin and, in our bioassay system, the receptor-mediated release of EDRF and prostacyclin are coupled (DeNucci *et al.*, 1988). Moreover, unlike L-Arg or L-HOArg, HOG is not a substrate for the constitutive NO

synthase present in EC showing that the effects of HOG are not due to an increased generation of NO by endothelial cells. The responses to GTN, which is metabolized to NO by vascular smooth muscle cells (Feelish & Kelm, 1991; Salvemini *et al.*, 1992), were not affected by HOG demonstrating that HOG does not release an EC-derived factor potentiating the action of NO. Clearly, both flow- and agonist-triggered relaxations were more stable down the cascade in the presence of HOG than in its absence.

The results summarized above exclude pharmacological activity of HOG on endothelial cells and all strongly suggest a chemical interaction between HOG and NO, either flow-induced or agonist-released from EC, leading to the formation of a novel potent vasodilator. This notion is further supported by the potentiation and stabilization by HOG of the vasorelaxant activity of authentic NO in the absence of EC and the formation of a more stable, chromatographically distinct vasodilator following reaction of HOG with NO in a test tube. The formation of this stable vasodilator in a test tube clearly shows that the reaction between HOG and NO is not an artefact of cultured cells or microcarrier beads.

HOG infused o.t. did not potentiate the relaxations of the first two detector tissues induced by EDRF or NO. However, a slight potentiation could be observed on the third detector tissue which was separated from the first one by 6 s. Infusion of HOG t.c., which markedly potentiated the vasorelaxant activity of EDRF or NO, further increased the time of interaction of HOG with EDRF or NO to 15–20 s, equivalent to the transit time of the perfusate through the column. Thus, the effects of HOG were proportional to the time of interaction between HOG and NO, indicating that the chemical reaction between HOG and NO is not instantaneous but requires some time (seconds) to occur.

Recently we have shown (Zembowicz *et al.*, 1991) that L-HOArg, an intermediate in the oxygenation of L-Arg to NO by the inducible NO synthase from macrophages (Stuehr *et al.*, 1991), is also a substrate for the constitutive endothelial NO synthase and that it is preferentially used by this enzyme despite saturating concentrations of L-Arg. Moreover, both L-HOArg and D-HOArg react with NO to form a more stable vasodilator with similar physicochemical properties to those of the HOG/NO adduct. Thus, the hydroxyguanidino moiety of L-HOArg is essential for its reactivity with NO and other hydroxyguanidino-derivatives should have similar properties. We have also shown (Zembowicz *et al.*, 1991) that, unlike L-Arg, L-HOArg causes EC-dependent relaxations which are resistant to the blockade by the inhibitors of NO synthase. In contrast, relaxant responses to HOG were completely abolished by L-NAME, demonstrating that they solely represent the potentiation of the vasorelaxant

activity of EDRF released by flow (shear-stress), a process abrogated by inhibitors of NO synthase (Hutcheson & Griffith, 1991). In our previous study we have shown that the relaxant activity of flow-induced EDRF was only little affected by D-HOArg. This may be best explained by the differences in experimental conditions. In the present experiments, we used larger numbers (9×10^7 vs 6×10^7) and volumes (3 vs 2 ml) of EC which resulted in greater production of NO and increased its reaction time with HOG, hence allowing for more efficient formation of the HOG/NO adduct.

There has been much debate as to whether EDRF is NO or a closely related compound. Earlier evidence for the chemical identity of EDRF and NO (Hutchinson *et al.*, 1987; Radomski *et al.*, 1987; Palmer *et al.*, 1987) was questioned after the demonstration of poor correlation of the biological activity and chemiluminescence or EPR signal of authentic NO when compared with those of EDRF released from cultured bovine endothelial cells (Myers *et al.*, 1989, 1990; Rubanyi *et al.*, 1991). These authors suggested that EDRF more closely resembles a S-nitrosothiol than NO. This debate will probably continue until a direct chemical method for the selective detection of NO has been developed. The chemical reaction of the hydroxyguanidino moiety with NO suggests one possible approach towards the development of such a method, i.e. monitoring of the formation of the stable HOG/adduct by u.v. photometry. More importantly, our results are consistent with the chemical identity of EDRF with NO.

HOG was without effect on the phenylephrine-induced contractions and acetylcholine-induced relaxations of intact rabbit aortic rings, hence indicating that HOG does not potentiate the biological activity of EDRF released from an intact endothelium by flow or agonists. Moreover, HOG does not have a hypotensive effect in the anaesthetized rat (Dr C. Theimermann, unpublished observation). This indicates that the diffusion time for NO between the endothelial NO synthase and vascular smooth muscle guanylate cyclase is too short for the efficient reaction with HOG. This explanation is consistent with the lack of effect of HOG given o.t. in the bioassay cascade.

We conclude that the hydroxyguanidino moiety of L-HOArg is essential for its reaction with NO and that EDRF released from endothelial cells by flow or agonist-mediated cell activation has the chemical characteristics of nitric oxide.

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References

- BILLIAR, T.R., CURRAN, R.D., STUEHR, D.J., STADLER, J., SIMMONS, R.L. & MURRAY, S.A. (1990). Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. *Biochem. Biophys. Res. Commun.*, **168**, 1034–1040.
- BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthase, a calmodulin requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.
- BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.
- DENUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor mediated release of endothelium-derived relaxing factor and prostacyclin from bovine endothelial cells are coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2334–2337.
- FEELISH, M. & KELM, M. (1991). Biotransformation of organic nitrates to nitric oxide by vascular smooth muscle and endothelial cells. *Biochem. Biophys. Res. Commun.*, **180**, 286–293.
- FORSTERMANN, U., GORSKY, L.D., POLLOCK, J.S., SCHMIDT, H.H.W., HELLER, M. & MURAD, F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1788–1792.
- GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine endothelial cells. *Br. J. Pharmacol.*, **87**, 685–694.
- HARPER, J.F. & BROOKER, G. (1975). Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.*, **1**, 207–218.

- HUTCHESON, I.R. & GRIFFITH, T.M. (1991). Release of endothelium-derived relaxing factor is modulated both by frequency and amplitude of pulsatile flow. *Am. J. Physiol.*, **261** (*Heart. Circ. Physiol.*, **30**), H257–H262.
- HUTCHINSON, P.J.A., PALMER, R.M.J. & MONCADA, S. (1987). Comparative pharmacology of EDRF and nitric oxide on vascular strips. *Eur. J. Pharmacol.*, **141**, 445–451.
- KWON, N.S., NATHAN, C.F., GILKER, C., GRIFFITH, O.W., MATTHEWS, D.E. & STUEHR, D.J. (1990). L-Citrullin production from L-arginine by macrophage nitric oxide synthase: the ureido oxygen derives from dioxygen. *J. Biol. Chem.*, **265**, 13442–13445.
- MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.
- MULSH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^G-[immino (nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 143–147.
- MYERS, P.R., GUERRA, R. & HARRISON, D.G. (1989). Release of NO and EDRF from cultured bovine endothelial cells. *Am. J. Physiol.*, **256** (*Heart Circ. Physiol.*, **25**), H1030–H1037.
- MYERS, P.R., MINOR, R.L., GUERRA, R., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.
- NATHAN, C.F. & HIBBS, J.B. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opinion Immunol.*, **3**, 65–70.
- PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.
- PALMER, R.M.J., FERRIDGE, A.G. & MONCADA, S. (1987). Nitric oxide release from vascular endothelial cells accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.*, **92**, 181–187.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.
- REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.*, **101**, 746–752.
- RUBANYI, G.M., JOHNS, A., WILCOX, D., BATES, F.N. & HARRISON, D.G. (1991). Evidence that s-nitrosothiol, but not nitric oxide, may be identical with endothelium-derived relaxing factor. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S41–S45.
- SALMON, J.A. (1978). A radioimmunoassay for 6-keto-prostaglandin F_{2α}. *Prostaglandins*, **15**, 383–397.
- SALVEMINI, D., MOLLACE, V., PISTELLI, A., ANGGARD, E.E. & VANE, J.R. (1992). Metabolism of glyceryl trinitrate to nitric oxide by endothelial cells and smooth muscle cells and its induction by *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 982–986.
- SNYDER, S.H. & BREDT, D.S. (1991). Nitric oxide as a neuronal messenger. *Trends Pharmacol. Sci.*, **12**, 125–128.
- STUEHR, D.J., KWON, N.S., NATHAN, C.F. & GRIFFITH, O.W. (1991). N^G-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.*, **266**, 6259–6263.
- VANE, J.R., ANGGARD, E.E. & BOTTING, R.M. (1990). Regulatory functions of the vascular endothelium. *N. Engl. J. Med.*, **323**, 27–36.
- WALKER, J.B. & WALKER, M.S. (1959). The enzymatic reduction of hydroxyguanidine. *J. Biol. Chem.*, **234**, 1481–1484.
- ZEMBOWICZ, A., HECKER, M., MACARTHUR, H., SESSA, W. & VANE, J.R. (1991). Nitric oxide and another potent vasodilator are formed from N^G-hydroxy-L-arginine by cultured endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11172–11176.

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