Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11172–11176, December 1991
Pharmacology

Nitric oxide and another potent vasodilator are formed from N^G-hydroxy-L-arginine by cultured endothelial cells

(endothelium-derived relaxing factor/arginine analogues/bioassay)

ARTUR ZEMBOWICZ*, MARKUS HECKER†, HEATHER MACARTHUR, WILLIAM C. SESSA‡, AND JOHN R. VANE

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

Contributed by John R. Vane, August 26, 1991

ABSTRACT **The hypothesis was investigated that N^G-hydroxy-L-arginine (L-HOArg) is an intermediate in the biosynthesis of nitric oxide (·NO) from L-arginine (L-Arg) by the constitutive ·NO synthase (NOS) present in endothelial cells (ECs). When infused through a column of bovine aortic ECs on beads, either L-HOArg or D-HOArg (1–10 μM) substantially potentiated relaxations of the bioassay tissues to ·NO released from the cells by ADP or bradykinin, and this effect was abolished by coinfusions of N^G-nitro-L-arginine (L-NO_2Arg) methyl ester (10 μM) or N^G-monomethyl-L-arginine (L-MeArg; 30 μM). Both L-HOArg and D-HOArg, irrespective of the presence of ECs, also potentiated relaxations induced by authentic ·NO, but not glyceryl trinitrate. This was due to a rapid chemical reaction of either isomer with ·NO, resulting in the formation of a potent and more stable vasodilator. When infusions of L-HOArg (3 μM) were consequently made in the presence of D-HOArg (10 μM), the L-isomer no longer had any effect on relaxations induced by authentic ·NO, but significantly increased the stimulated release of ·NO from the column of ECs. The conclusion that L-HOArg is a substrate for the constitutive NOS in cultured ECs was strongly supported by the L-NO_2Arg-sensitive conversion of L-HOArg, but not D-HOArg, to ·NO by NOS preparations from these cells. Interestingly, cultured ECs produced from L-HOArg (≥3 μM), but not D-HOArg, a stable vasodilator, the effects of which were inhibited by oxyhemoglobin (0.3–3 μM). However, the formation of this substance was not prevented by L-NO_2Arg methyl ester (10 μM) or L-MeArg (10–100 μM), suggesting an enzymatic pathway different from NOS.**

Endothelium-derived relaxing factor (EDRF) or nitric oxide (·NO; ref. 1) is a potent vasodilator and antithrombotic agent (2). ·NO also plays a role in the cytotoxicity of activated macrophages and as a neurotransmitter (3, 4). The formation of ·NO from L-arginine (L-Arg) is catalyzed by a NADPH-dependent dioxygenase (5), referred to as ·NO synthase (NOS), which can exist in at least two distinct forms, a constitutive agonist-triggered and calcium/calmodulin-dependent NOS, which is mainly present in neuronal cells (6) and vascular endothelial cells (ECs; ref. 7), and a calcium-independent inducible NOS, which is found predominantly in cytokine-activated macrophages (8) and smooth muscle cells (9). The exact mechanism by which these enzymes convert L-Arg to ·NO is not known. It has been postulated (8) and recently demonstrated (10) that N^G-hydroxy-L-arginine (L-HOArg) is an intermediate in the biosynthesis of ·NO by the inducible NOS from activated macrophages. Consequently, we have postulated that L-HOArg is an intermediate in the biosynthesis of ·NO by the constitutive NOS present in ECs. Here, we demonstrate (*i*) that L-HOArg is a substrate for this enzyme, (*ii*) that cultured ECs produce a vasodilator substance from L-HOArg, which is distinct from ·NO, and (*iii*) that both L-HOArg and D-HOArg react chemically with authentic ·NO or EC-derived ·NO to form a potent and more stable vasodilator.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. Cytochrome *c* (from bovine heart), N^G-nitro-L-arginine (L-NO_2Arg) methyl ester, superoxide dismutase (SOD; from bovine erythrocytes), and xanthine oxidase (from milk) were obtained from Sigma; N^G-monomethyl-L-arginine (L-MeArg) acetate salt was from Calbiochem; L-NO_2Arg acetate salt was from Bachem; (6*R*,*S*)-5,6,7,8-tetrahydro-L-biopterin was from B. Schirck's Laboratories (Jona, Switzerland); and glyceryl trinitrate (GTN or Nitronal) was from Lipha Pharmaceuticals (West Drayton, U.K.). 9,11-Dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) was a generous gift from Upjohn. L-HOArg and D-HOArg (purity ≥98%, contamination by hydroxylamine ≤0.1%) (11) were synthesized by Paul L. Feldman (Glaxo Research Institute, Research Triangle Park, NC). Saturated solutions of ·NO (1–3 mM) were prepared by bubbling 25 ml of ·NO gas (Merck) into 5 ml of helium-deoxygenated water kept in an air-tight flask at 0–4°C. All other reagents and solvents were of the highest commercially available quality from either Sigma or Merck.

Cell Culture. Bovine aortic ECs were harvested and grown on Cytodex-3 microcarrier beads (Pharmacia/LKB) in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (vol/vol) fetal calf serum (GIBCO) as described in ref. 12. LLC-PK_1 pig kidney epithelial cells (ATCC CL 101) were obtained from the European Collection of Animal Cell Cultures and grown on 12-well plates in medium 199 (Flow Laboratories) supplemented with 10% fetal calf serum.

Bioassay of ·NO. Approximately 6×10^7 ECs on beads were packed into a jacketed chromatography column and perfused at 5 ml/min with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (12) containing 5.6 μM indomethacin and in some experiments SOD (10–30 units/ml). The effluent from the column superfused a cascade (13) of three or four spirally cut strips of rabbit aorta (RbA), which were denuded

Abbreviations: ·NO, nitric oxide; SOD, superoxide dismutase; L-MeArg, N^G-monomethyl-L-arginine; L-NO_2Arg, N^G-nitro-L-arginine; L-HOArg, N^G-hydroxy-L-arginine; D-HOArg, N^G-hydroxy-D-arginine; NOS, ·NO synthase; EC, endothelial cell; EDRF, endothelium-derived relaxing factor; GTN, glyceryl trinitrate; RbA, rabbit aorta; t.c., through the column of ECs; o.t., over the bioassay tissue.

*On leave from the Department of Pharmacology, N. Copernicus Academy of Medicine, Grzegorzecka 16, 31-531 Cracow, Poland.
†To whom reprint requests should be addressed at: the Institute of Applied Physiology, University of Frieburg, Herman-Herder Strasse 7, D-W-7800 Frieburg, Federal Republic of Germany.
‡Present address: Department of Pharmacology, University of Virginia Health Science Center, School of Medicine, P.O. Box 325, Charlottesville, VA 22908.

of endothelium and preconstricted with 30 nM U46619. Their relaxations were recorded as described in ref. 12.

Determination of Superoxide Anion (O_2^-) Production. O_2^- formation was determined spectrophotometrically by monitoring the reduction of cytochrome *c* (10 μM) at 550 nm vs. time in a cuvette at 37°C containing Dulbecco's phosphate-buffered saline (DPBS; with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) at pH 7.4, xanthine (100 μM), and xanthine oxidase (21 milliunits/ml). An extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ (14) was used to calculate the rate of O_2^- production. SOD (10 units/ml) or L-HOArg (10–1000 μM) were preincubated for 2 min with xanthine oxidase before the reaction was initiated by the addition of xanthine.

Preparation of Subcellular Fractions from Cultured ECs. All of the following procedures were carried out at 0–4°C. Approximately 1×10^9 ECs on beads were suspended in 50 mM Tris·HCl (pH 7.4) containing 10 mM EDTA, 5 mM glucose, 1.15% (wt/vol) KCl, 0.1 mM DL-dithiothreitol, leupeptin (2 mg/liter), pepstatin A (2 mg/liter), trypsin inhibitor (10 mg/liter), and phenylmethylsulfonyl fluoride (44 mg/liter). The cell suspension was bubbled with helium for 15 min and sonicated, and the homogenate was centrifuged in three subsequent steps at 1000 × *g*, 10,000 × *g*, and 200,000 × *g* for 10, 20, and 30 min, respectively. The 200,000 × *g* supernatant (cytosol) was concentrated by using disposable Centricon-10 filters (molecular weight cut-off 10,000; from Amicon), and the 200,000 × *g* pellet (microsomes) was resuspended in 50 mM Tris·HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, leupeptin (2 mg/liter), pepstatin A (2 mg/liter), trypsin inhibitor (10 mg/liter), phenylmethylsulfonyl fluoride (44 mg/liter), and 10% (vol/vol) glycerol. Protein concentrations were determined by using Peterson's modification of the micro-Lowry method (15).

Determination of NOS Activity. Samples of the cytosol or membrane fraction (50 μg of protein) were diluted in 1 ml of DPBS containing 100 μM NADPH, 100 μM dithiothreitol, 1 μM (6*R*,*S*)-5,6,7,8-tetrahydro-L-biopterin, 0.1 μM calmodulin, and SOD (100 units/ml) and incubated for 10 min at 37°C in the presence or absence of L-Arg, L-HOArg, D-Arg, or D-HOArg (all at 100 μM) with monolayers of LLC-PK_1 cells in 12-well plates (5×10^5 cells per well) pretreated for 30 min with 0.5 mM isobutylmethylxanthine. In some experiments, 100 μM L-NO_2Arg was preincubated with the microsomal fraction for 5 min. Intracellular cGMP levels were determined after aspiration of the supernatant, extraction of the cells with ice-cold 5% (wt/vol) trichloroacetic acid, and centrifugation at 10,000 × *g* for 10 min. Trichloroacetic acid was removed from the supernatant with 2 vol of 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane. The concentration of cGMP in the aqueous phase was measured by using a radioimmunoassay developed in our laboratory using a specific antiserum, kindly provided by Hans Strobach (Institute of Pharmacology, University of Düsseldorf) and ^{125}I-labeled cGMP from Amersham. The detection limit of the assay was 0.2 pmol/100 μl of sample and the cross-reactivity with cAMP was <0.001%.

HPLC Analysis of L-HOArg and Its Reaction Product with ·NO. Normal-phase HPLC analysis of the reaction of L-HOArg with ·NO was carried out by using a 250 × 4.6 (i.d.) mm Ultra-Techsphere NH_2 (5 μm) HPLC column (HPLC Technology), which was isocratically eluted at 1.5 ml/min with 40% (vol/vol) 10 mM KH_2PO_4, pH 4.3/60% (vol/vol) acetonitrile/water, 50:7 (vol/vol). The UV-absorbance of the effluent was continuously recorded at 205 nm. L-Citrulline, L-Arg, and L-HOArg were eluted at 4.0, 6.0, and 8.0 min, respectively.

Statistical Analysis. All values in the figures and text are expressed as mean ± SEM of *n* observations. A one-way analysis of variance followed by a Bonferroni test or a one-sample two-tailed *t* test was used, where appropriate, to assess the statistical significance of results. A *P* value of <0.05 was considered statistically significant.

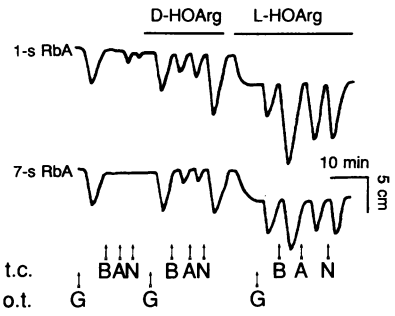

FIG. 1. L-HOArg and D-HOArg potentiate the relaxant activity of EC-derived ·NO. The response (representative of four experiments) of the first and third of the four bioassay tissues (RbAs) is shown. Relaxations induced by bradykinin (B, 20 pmol), ADP (A, 10 nmol), or authentic ·NO (N, 1–3 nmol) injected t.c. were markedly potentiated and stabilized in the presence of D-HOArg (10 μM) or L-HOArg (10 μM); infused t.c. L-HOArg was more potent than D-HOArg in potentiating the effects of bradykinin (4.1 ± 1.1 times) or ADP (3.4 ± 1.2 times) but not ·NO (1.0 ± 0.1 times) ($n = 4$). L-HOArg but not D-HOArg, when infused t.c., also caused a relaxation that was stable down the cascade. L-HOArg or D-HOArg did not affect the relaxant responses to GTN (G, 20 pmol) given o.t. Note these and the bioassay experiments depicted in Figs. 2–5 were performed in the absence of SOD, thus precluding the detection of the flow-induced release of ·NO.

RESULTS

L-HOArg Elicits the Release from ECs of a Relaxing Factor Different from ·NO. Infusions of L-HOArg or D-HOArg (1–20 μM) over the bioassay tissues (o.t.) had no direct effect on their tone. However, when infused through the column of ECs (t.c.), L-HOArg, but not D-HOArg, at concentrations >1 μM caused a relaxation of the bioassay tissues that, unlike that for authentic ·NO, did not diminish down the cascade (cf. Figs. 1–3). The relaxation provoked by L-HOArg waned on stopping the infusion and was not observed when empty beads were used instead of EC (Fig. 4). When L-HOArg (10 μM) was infused t.c. and a 2.5-min delay coil was inserted between the second and third bioassay tissue, the relaxing factor released from the EC column was still able to cause a

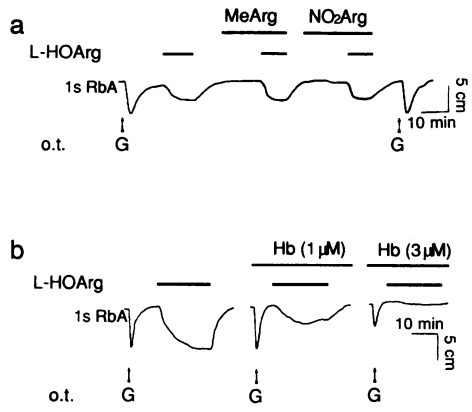

FIG. 2. L-HOArg-induced EC-dependent relaxations are inhibited by oxyhemoglobin but not by inhibitors of ·NO biosynthesis. The responses of the first of four bioassay tissues from two independent experiments are shown. L-HOArg (10 μM, t.c.) caused a relaxation of the bioassay tissue that was not inhibited by MeArg (100 μM, t.c.) or L-NO_2Arg methyl ester (10 μM, t.c.) infused for 20 min (*a*). However, the relaxant effect of L-HOArg was blocked by increasing concentrations of oxyhemoglobin (Hb, t.c.) (*b*). Representative traces of four (*a*) and three (*b*) experiments are shown.

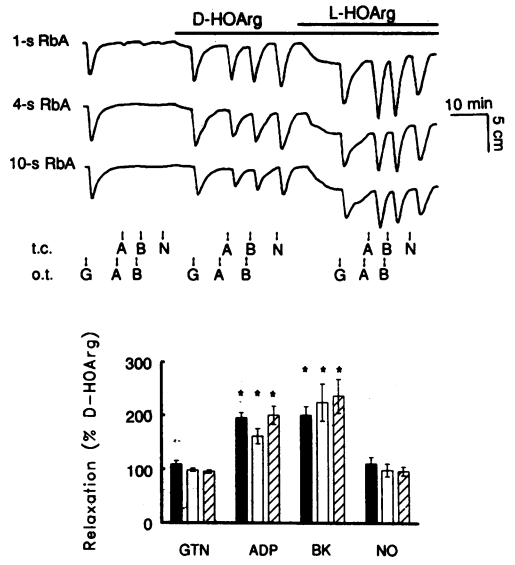

FIG. 3. L-HOArg potentiates the agonist-triggered release of ·NO from ECs. (*Upper*) Response of the first, second, and fourth detector tissues. Infusion of D-HOArg (10 μM, t.c.) had only a weak effect on the tone of the bioassay tissues but potentiated and stabilized relaxations induced by ADP (A, 5 nmol, t.c.), bradykinin (B, 5 pmol, t.c.), or authentic ·NO (N, 1–3 nmol, t.c.). When L-HOArg (3 μM) was infused t.c. in the presence of D-HOArg, it caused a nongraded relaxation and further potentiated relaxations induced by ADP or bradykinin but not those induced by authentic ·NO. Responses to GTN (G, 90 pmol, o.t.) were not affected by D-HOArg or L-HOArg. The results of four experiments are summarized (*Lower*) showing relaxations of the first (solid bars), second (open bars), and fourth (hatched bars) detector tissues induced by GTN, ADP, bradykinin, or authentic ·NO during infusions of L-HOArg in the presence of D-HOArg. Values (mean ± SEM) are expressed as percent of the relaxant responses in the presence of D-HOArg alone.
*$P < 0.05$ when compared to 100 by a one-sample two-tailed t test.

significant relaxation of the two tissues below the coil, amounting to 41 ± 3% of the relaxation of the tissues above the coil ($n = 4$, data not shown). Interestingly, the formation of this stable relaxing factor from L-HOArg was not blocked by inhibitors of ·NO biosynthesis (Fig. 2), such as L-MeArg (10 μM) or L-NO_2Arg methyl ester (10–100 μM), in concentrations sufficient completely to inhibit the flow-induced or agonist-triggered release of ·NO from the column of ECs (cf. Fig. 5). In contrast, the ·NO scavenger oxyhemoglobin (0.3–3

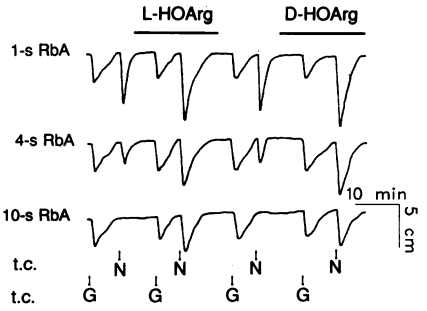

FIG. 4. Stabilization of the relaxant activity of ·NO by HOArg is independent of the presence of ECs. The response of the first, second, and fourth bioassay tissues superfused in a column of empty beads is shown. Authentic ·NO (N, 1–3 nmol) injected t.c. caused a relaxant response that disappeared down the cascade. This response was clearly potentiated and more stable down the cascade when L-HOArg (10 μM) or D-HOArg (10 μM) was infused t.c. Infusion of L-HOArg or D-HOArg had no effect on the relaxant responses to GTN (G, 45 pmol, o.t.). Representative traces of three experiments are shown.

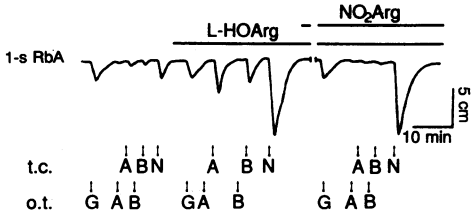

FIG. 5. L-NO_2Arg inhibits the potentiation by L-HOArg of the relaxation induced by EC-derived ·NO but not authentic ·NO. The response of the first of four bioassay tissues is shown. Infusion of L-HOArg (1 μM, t.c.) markedly potentiated relaxations induced by ADP (A, 5 nmol, t.c.) or bradykinin (B, 5 pmol, t.c.), as well as those induced by authentic ·NO (N, 1–3 nmol, t.c.). In the presence of L-NO_2Arg methyl ester (10 μM) infused t.c. for 20 min, the effect of L-HOArg on the relaxant responses to ADP or bradykinin was abolished but relaxations induced by authentic ·NO remained unchanged. L-HOArg or L-NO_2Arg methyl ester did not affect the relaxant responses to GTN (G, 22.5 pmol, o.t.). Representative trace of four experiments is shown.

μM) dose-dependently inhibited L-HOArg-induced relaxations (Fig. 2).

L-HOArg and D-HOArg Potentiate the Relaxant Activity of ·NO Due to the Formation of a More Stable Relaxing Factor. When infused t.c., but not o.t., both L-HOArg (1–20 μM, $n = 14$) and D-HOArg (1–10 μM, $n = 13$) substantially potentiated relaxations of the bioassay tissues to ·NO released from the column of ECs after injections t.c. of bradykinin or ADP. This effect was dose-dependent for both isomers with L-HOArg being 4-fold more potent than D-HOArg (cf. Fig. 1). Similar to the relaxation induced by t.c. infusions of L-HOArg, these relaxations were clearly more stable down the cascade (half-life, >>10 s) than those induced by authentic or EC-derived ·NO in the absence of L-HOArg or D-HOArg (half-life, ≤3 s). Moreover, the potentiation by L-HOArg or D-HOArg of relaxations induced by bradykinin or ADP was abolished by L-MeArg (30 μM, $n = 3$) or L-NO_2Arg methyl ester (10 μM, $n = 5$), whereas those elicited by authentic ·NO were unaffected (Fig. 5).

When empty microcarrier beads were used instead of ECs, both L-HOArg and D-HOArg still potentiated relaxations induced by authentic ·NO, but not GTN (Fig. 4). This effect was especially pronounced on the third and fourth bioassay tissues, indicating that both isomers prevent the degradation of ·NO in the cascade bioassay system.

Apparently, this was not due to scavenging O_2^-, for in the presence of SOD (10–30 units/ml) infused t.c., virtually identical bioassay results were obtained; i.e., L-HOArg (1–10 μM) or D-HOArg (1–20 μM) had no direct effect on the tone of the bioassay tissues ($n = 3$), L-HOArg (1–20 μM t.c., $n = 10$), but not D-HOArg (1–100 μM t.c., $n = 4$), caused a nongraded relaxation, whereas both L-HOArg (1–20 μM t.c., $n = 10$) and D-HOArg (1–10 μM t.c., $n = 4$) potentiated relaxations induced by EC-derived ·NO as well as authentic ·NO, and the latter effect was also observed with empty microcarrier beads ($n = 2$, data not shown). Moreover, L-HOArg at concentrations up to 1 mM did not inhibit the O_2^--dependent reduction of cytochrome c by xanthine/xanthine oxidase [control, 10.4 ± 1.4 nmol of O_2^- per min ($n = 7$); L-HOArg (10 μM), 13.6 ± 3.4 nmol of O_2^- per min ($n = 3$)], whereas SOD at 10 units/ml was clearly active (1.9 ± 0.1 nmol of O_2^- per min; $n = 3$, $P < 0.05$).

However, when incubated in deoxygenated water at 0–4°C with authentic ·NO, both L-HOArg and D-HOArg were converted to a chromatographically distinct compound that was eluted close to L-Arg (Fig. 6). This HPLC fraction, when injected o.t. in the cascade bioassay system, elicited a stable relaxation of the bioassay tissues that was strikingly similar to that induced by authentic ·NO in the presence of t.c.

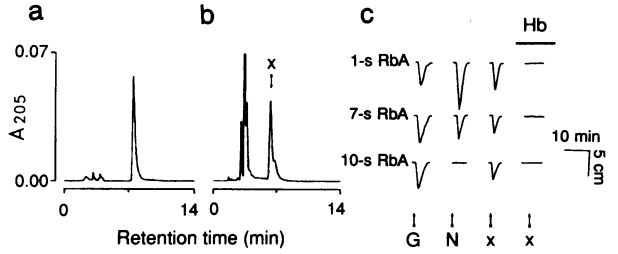

FIG. 6. L-HOArg reacts with ·NO to form a chromatographically distinct vasodilator. (*a*) Typical normal-phase HPLC chromatogram of L-HOArg (10 nmol) eluting at 8.0 min. (*b*) Chromatogram of a sample (1 μl corresponding to 10 nmol of L-HOArg) of the same solution of L-HOArg (10 mM, in deoxygenated water; total volume, 0.5 ml) after a 120-min incubation in the presence of ·NO (5 ml of ·NO gas) at 0–4°C. L-HOArg was almost quantitatively converted to a compound (x) that eluted at 6.5 min. The two major peaks before compound x represent NO_3^- (retention time, 3.4 min) and NO_2^- (retention time, 3.7 min), respectively, breakdown products of ·NO in water. The fraction corresponding to compound x was collected and, when injected o.t. (5 μl corresponding to ≈1 nmol) in the cascade bioassay, caused a stable relaxation of all detector tissues (*c*). This effect was abolished when oxyhemoglobin (Hb, 10 μM) was concomitantly infused o.t. For comparison, relaxations of the same tissues induced by GTN (G, 90 nmol, o.t.) and authentic ·NO (N, 1–3 nmol, o.t.) are also shown.

infusions of L-HOArg or D-HOArg and was abolished by infusions o.t. of oxyhemoglobin (10 μM, Fig. 6). Both the HPLC band and the biological activity rapidly (minutes) disappeared in oxygenated water at room temperature, and according to chemiluminescence analysis, the isolated compound readily released ·NO when injected into water at temperatures exceeding 60°C.

L-HOArg Is a Substrate for EC NOS. As the chemical reaction of HOArg with ·NO occurred with either isomer, the effect of L-HOArg on the release of ·NO from ECs was investigated by infusing low concentrations of this isomer (3 μM) in the presence of high concentrations of D-HOArg (10 μM). Under these conditions, L-HOArg clearly potentiated (2-fold) relaxations induced by ·NO released from the column of ECs by ADP or bradykinin (Fig. 3). However, the relaxant responses to GTN and, more importantly, authentic ·NO were not affected, indicating that the effects of L-HOArg were not attributable to an increase of the overall capacity of HOArg to react with ·NO.

To substantiate the conclusion that L-HOArg is converted to ·NO by constitutive NOS in ECs, subcellular fractions were prepared from cultured ECs and tested for NOS activity. Constitutive enzyme activity was largely recovered (80–90%) in the membrane fraction, which was subsequently employed for the NOS assay. Either L-HOArg or L-Arg (100 μM), but not D-HOArg or D-Arg, caused a 2- to 3-fold increase in the formation of cGMP in LLC-PK_1 cells, which was completely inhibited by equimolar concentrations of L-NO_2Arg (Fig. 7).

DISCUSSION

L-HOArg is a substrate and possibly the intermediate in the biosynthesis of ·NO by the inducible NOS from activated macrophages (8). We have investigated the hypothesis that L-HOArg is also a substrate for the constitutive NOS in ECs and, hence, potentiates the release of ·NO from these cells. Surprisingly, we found that both isomers of HOArg, irrespective of the presence of ECs, potentiate the relaxant effects of authentic ·NO, but not GTN, suggesting the involvement of a nonenzymatic process. Moreover, in the absence of SOD, the half-life of ·NO down the tissue cascade

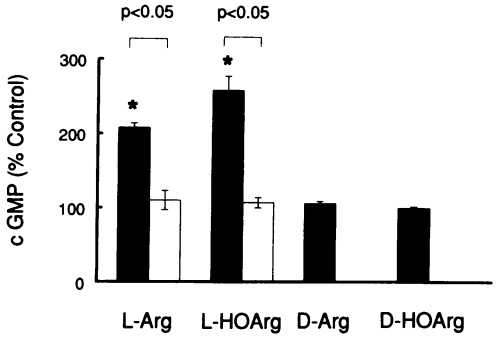

FIG. 7. L-HOArg is a substrate for EC NOS. Incubation of the microsomal protein (50 μg) with L-Arg (100 μM, $n = 8$) or L-HOArg (100 μM, $n = 8$) caused a significant increase in LLC-PKC_1 cell intracellular cGMP. These effects were abolished by coincubations with L-NO_2Arg (100 μM, $n = 4$; Bonferroni $P < 0.05$; open bars). In the presence of D-Arg (100 μM, $n = 4$) or D-HOArg (100 μM, $n = 3$) no increase in cGMP was observed. Results (mean ± SEM) are expressed as percentage of control cGMP levels in cells incubated over the same period of time.
*$P < 0.05$ when compared to 100 by a one-sample two-tailed t test. Note that L-Arg (111 ± 7%, $n = 3$) and L-HOArg (110 ± 4%, $n = 4$) alone had no significant effect on LLC-PK_1 cell cGMP levels.

was ≤3 s, whereas in the presence of L-HOArg or D-HOArg, the half-life of ·NO was significantly increased (>>10 s).

One possibility we considered was that HOArg scavenges O_2^-, thereby stabilizing ·NO (16). However, the same bioassay results were obtained in the absence or presence of SOD, and L-HOArg did not inhibit the generation of O_2^- by xanthine/xanthine oxidase, whereas SOD was clearly active, thus favoring the alternative, a chemical reaction of L-HOArg or D-HOArg with ·NO. This hypothesis was strongly supported by the following findings: (*i*) HPLC analysis of the reaction products of L-HOArg and ·NO revealed the quantitative formation of a labile (upon exposure to oxygen) compound; (*ii*) the corresponding HPLC fraction had the expected vasorelaxant properties; (*iii*) the biological activity of this substance was inhibited by oxyhemoglobin; and (*iv*) it readily released ·NO in water at elevated temperatures. All these characteristics point to a chemical reaction of ·NO with L-HOArg or D-HOArg, resulting in the formation of a considerably more stable adduct. Apparently, this reaction requires some time (seconds), for the effects of L-HOArg or D-HOArg on relaxations induced by ·NO injected t.c. (transit time, 20–30 s) were more pronounced when compared to those induced by ·NO released from the column of ECs (transit time, ≤10 s) (cf. Figs. 1 and 5). This may also explain the lack of effect of o.t. infusions of either isomer. The relative potency of the HOArg-NO adduct is difficult to judge on the basis of the present data, but it can be inferred from Fig. 6 that it is at least as potent as ·NO itself and clearly much more stable.

Were this chemical reaction the only mechanism for the potentiation of the effects of ·NO released from ECs, both isomers would be equipotent. However, L-HOArg was clearly more potent in this respect than D-HOArg, indicating that the L-isomer increased the biosynthesis of ·NO. This conclusion was based on the potentiation by L-HOArg of the agonist-triggered release of ·NO in the presence of a high concentration of D-HOArg without affecting relaxant responses to authentic ·NO; and the conversion of L-HOArg, but not D-HOArg, to ·NO by the constitutive membrane-bound NOS prepared from cultured ECs.

Recently Wallace *et al.* (17) reported on the vasorelaxant activity of L-HOArg in ring preparations from bovine intrapulmonary artery incubated in Krebs solution for 14 h. Although the observed inhibition of L-HOArg-induced relax-

ations by oxyhemoglobin, L-MeArg, or L-NO_2Arg methyl ester is consistent with L-HOArg being metabolized to ·NO, the enzyme responsible for this effect is more likely to be the inducible rather than the constitutive NOS, elicited by the low levels of endotoxin sometimes present in Krebs solution (18). Moreover, since L-HOArg was not tested in comparison with D-HOArg, the relative importance of the other mechanisms contributing to the vasodilator activity of L-HOArg, as demonstrated in the present study, cannot be judged. Furthermore, data on the purity of the synthesized L-HOArg were not provided, which is important, for high concentrations (≥100 μM) of L-HOArg or D-HOArg can relax vascular preparations due to the presence of small amounts of hydroxylamine (NH_2OH).

The agonist-triggered biosynthesis of ·NO by cultured ECs depends on the availability of intracellular free L-Arg (19). However, L-Arg does not normally potentiate the release of ·NO from these cells, because the intracellular concentration of L-Arg in cultured ECs (100–200 μM) is well above the K_m of NOS (1–10 μM; refs. 6 and 7) and these cells recycle L-Arg from L-citrulline, the by-product of ·NO biosynthesis, thereby maintaining their L-Arg levels (20). However, L-HOArg at very low concentrations (≥1 μM) significantly potentiated the stimulated release of ·NO from cultured ECs. As the uptake of L-HOArg or L-Arg by these cells is not different (data not shown), this finding suggests that the constitutive NOS present in ECs has a lower K_m or higher V_{max} for L-HOArg than L-Arg, consistent with the hypothesis that L-HOArg is an intermediate in the biosynthesis of ·NO by this enzyme. Clearly, the effects of L-HOArg cannot be attributed to a nonspecific activation of cultured ECs, for neither L-HOArg nor D-HOArg had any effect on the release of prostacyclin from these cells (data not shown).

Importantly, ECs produce from L-HOArg, but not from D-HOArg, another vasodilator distinct from ·NO due to its remarkable stability in the bioassay cascade. The generation of this substance was not affected by inhibitors of NOS, but its vasorelaxant effect was inhibited by oxyhemoglobin, implying an action based on the release of ·NO from this compound. We considered the possibility that ECs hydrolyze L-HOArg to L-citrulline and NH_2OH, a potent and stable vasodilator. However, we found no evidence for such a deiminase activity in cultured ECs, and relatively high concentrations of NH_2OH (≥1 μM) were needed to elicit a comparable relaxation (data not shown).

Another possibility is that L-HOArg is converted to ·NO by an enzymatic pathway distinct from the NO_2Arg- or MeArg-inhibitable NOS and that ·NO produced by this pathway reacts with excess L-HOArg upon release from the ECs to form the more stable adduct. There has been much debate as to whether EDRF is ·NO or a closely related compound [i.e., a *S*-nitrosothiol (21)], perhaps acting as a carrier molecule for ·NO. The endogenous formation of the HOArg-NO adduct would serve the function of such a carrier molecule, and this could be especially important for the flow-induced release of EDRF, which was little affected by D-HOArg, despite a strong potentiating effect on relaxations elicited by authentic ·NO.

In summary, we conclude that L-HOArg has three mechanisms of action. It is a substrate for EC NOS and is preferentially used by this enzyme, thus leading to an enhanced release of ·NO despite saturating concentrations of L-Arg. Moreover, ·NO reacts with HOArg to form a potent and more stable vasodilator. The conclusion that another EDRF is formed from L-HOArg warrants further investigation.

We are indebted to Dr. Paul L. Feldman for the generous supply of L-HOArg and D-HOArg, Drs. Garry J. Southan and Tomasz A. Swierkosz for their help with the chemiluminescence analysis and bioassay, respectively, Elizabeth G. Wood for the steady supply of LLC-PK_1 and endothelial cells, and Ivana Vojnovic and Desmond T. Walsh for expert technical assistance. This work was supported by a grant from Glaxo Group Research Ltd.

1. Palmer, R. J. M., Ferridge, A. G. & Moncada, S. (1987) *Nature (London)* **327**, 524–526.
2. Vane, J. R., Änggard, E. E. & Botting, R. M. (1990) *N. Engl. J. Med.* **323**, 27–36.
3. Nathan, C. F. & Hibbs, J. B. (1991) *Curr. Opinion Immunol.* **3**, 65–70.
4. Snyder, S. H. & Bredt, D. S. (1991) *Trends Pharmacol. Sci.* **12**, 125–128.
5. Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E. & Stuehr, D. J. (1990) *J. Biol. Chem.* **265**, 13442–13445.
6. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 682–685.
7. Förstermann, U., Pollock, J. S., Schmidt, H. H. H. W., Heller, M. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1788–1792.
8. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry* **27**, 8706–8711.
9. Hauschildt, S., Lückhoff, A., Mülsch, A., Kohler, J., Besslor, W. & Busse, R. (1990) *Biochem. J.* **270**, 351–356.
10. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* **266**, 6259–6263.
11. Feldman, P. L. (1991) *Tetrahedron Lett.* **32**, 875–878.
12. DeNucci, G., Gryglewski, R. J., Warner, T. D. & Vane, J. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2334–2338.
13. Gryglewski, R. J., Moncada, S. & Palmer, R. J. M. (1986) *Br. J. Pharmacol.* **87**, 685–694.
14. McCord, J. M. & Fridovitch, I. J. (1969) *J. Biol. Chem.* **244**, 6049–6055.
15. Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356.
16. Gryglewski, R. J., Moncada, S. & Palmer, R. M. J. (1986) *Nature (London)* **320**, 454–456.
17. Wallace, G. C., Gulati, P. & Fukuto, J. M. (1991) *Biochem. Biophys. Res. Commun.* **176**, 528–534.
18. Rees, D. D., Cellek, S., Palmer, R. M. J. & Moncada, S. (1990) *Biochem. Biophys. Res. Commun.* **173**, 541–547.
19. Mitchell, J. A., Hecker, M. & Vane, J. R. (1990) *Eur. J. Pharmacol.* **176**, 253–254.
20. Hecker, M., Sessa, W. C., Harris, H. J., Änggard, E. E. & Vane, J. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8612–8616.
21. Rubanyi, G. M., Johns, A., Wilcox, D., Bates, F. N. & Harrison, D. G. (1991) *J. Cardiovasc. Pharmacol.* **17**, Suppl. 3, S41–S45.