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Exogenous N^G-hydroxy-L-arginine causes nitrite production in vascular smooth muscle cells in the absence of nitric oxide synthase activity

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Abstract

Nitric oxide (NO) production from exogenous N^G-hydroxy-L-arginine (OH-L-Arg) was investigated in rat aortic smooth muscle cells in culture by measuring nitrite accumulation in the culture medium. As well, the interaction between OH-L-Arg and L-arginine uptake via the y^+ cationic amino acid transporter was studied. In cells without NO-synthase activity, OH-L-Arg (1–1000 μM) induced a dose-dependent nitrite production with a half-maximal effective concentration (EC_{50}) of 18.0 ± 1.5 μM (n = 4–7). This nitrite accumulation was not inhibited by the NO-synthase inhibitor N^G-nitro-L-arginine methyl ester, L-NAME (300 μM). In contrast, it was abolished by miconazole (100 μM), an inhibitor of cytochrome P_{450}. Incubation of vascular smooth muscle cells with LPS (10 μg/ml) induced an L-NAME inhibited nitrite accumulation, but did not enhance the OH-L-Arg induced nitrite production. OH-L-Arg and other cationic amino acids, L-lysine and L-ornithine, competitively inhibited [^{3}H]-L-arginine uptake in rat aortic smooth muscle cells, with inhibition constants of 195 ± 23 μM (n = 12), 260 ± 40 μM (n = 5) and 330 ± 10 μM (n = 5), respectively. These results show that OH-L-Arg is recognized by the cationic L-amino acid carrier present in vascular smooth muscle cells and can be oxidized to NO and nitrite in these cells in the absence of NO-synthase, probably by cytochrome P_{450} or by a reaction involving a cytochrome P_{450} by-product.

Key words: N^G-Hydroxy-L-arginine; Smooth muscle cell; Nitric oxide; NO-synthase; Arginine uptake

1. Introduction

N^G-hydroxy-L-arginine (OH-L-Arg) is a stable intermediate compound formed during the two-step enzymatic reaction leading to the production of nitric oxide (NO) and L-citrulline from L-arginine by NO-synthases [1,2,3]. It has been shown that OH-L-Arg is released into the extracellular medium by cells which possess an NO-synthase [2,4,5]. This suggests that OH-L-Arg might produce paracrine or endocrine effects, provided that it can enter into other cells once released in the extracellular fluid. The aim of the present investigation was to explore this possibility in vascular smooth muscle cells. It has been reported that OH-L-Arg is able to produce endothelium-dependent relaxation in blood vessels [6].

The role of NO in the regulation of vascular tone is well-documented (for review, see [7]). In the vessel wall, NO is produced by constitutive NO-synthases in endothelial cells and in some neurons, and diffuses to smooth muscle cells where it induces relaxation via activation of soluble guanylyl cyclase. Vascular smooth muscle cells do not contain a constitutive NO-synthase, but an inducible NO-synthase can be expressed in these cells following exposure to bacterial lipopolysaccharide (LPS; [8]) or cytokines [9].

We report here the effects of OH-L-Arg on NO production in cultured rat aortic smooth muscle cells in control conditions and after exposure to LPS and the associated induction of NO-synthase. NO production was assessed by monitoring the accumulation of nitrite, the stable breakdown product of NO, in the culture medium. Since a cationic amino acid carrier transporting L-arginine has been recently characterized in cultured rat aortic smooth muscle cells [10,11], the interaction of OH-L-Arg with this system was investigated by studying competition with [^{3}H]L-arginine uptake.

2. Materials and methods

2.1. Materials

Papain, bovine serum albumin, dithiothreitol, N^G-nitro-L-arginine methyl ester (L-NAME), L-ornithine, L-arginine, and ascorbic acid were obtained from Sigma (St. Louis, MI, USA). Hank's balanced salt solution (HBSS), Ham's F10, trypsin (1.5 g/l) and Eagle's minimum essential medium (MEM) were purchased from Eurobio (Les Ulis, France), Ultroser G (a defined serum substitute) from IBF (Villeneuve la Garenne, France), glutamine from Merck (Darmstadt, Germany). [^{3}H]L-arginine (58.4 Ci/mmol) was obtained from DuPont de Nemours (Bad Homburg, Germany), L-lysine from Calbiochem (San Diego, CA, USA) and LPS (*E. coli* 055: B5) from Difco (Detroit, MI, USA). OH-L-Arg, synthesized according to a previously described method [12], was kindly provided by Dr. J.L. Boucher (Laboratoire de Chimie

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Abbreviations: OH-L-Arg, N^G-hydroxy-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; HBSS, Hank's balanced salt solution; NO, nitric oxide.

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et Biochimie Pharmacologiques et Toxicologiques, CNRS URA 400, Paris, France). L-NAME, L-lysine, L-ornithine, L-arginine, LPS and OH-L-Arg solutions were freshly prepared before each experiment in MEM without arginine. Stock solutions of papain (1.5 mg/ml), bovine serum albumin (0.2%), and dithiothreitol (1 mM) were stored frozen.

2.2. Cell cultures

Vascular smooth muscle cells were obtained from thoracic aortae of male Wistar rats (9 weeks old) according to the method described by Clapp and Gurney [12] and used at the first passage. Briefly, aortae were dissected out, placed in HBSS, and connective tissue, endothelium and adventitia were mechanically removed. The medial layers were washed and incubated overnight at 4°C in HBSS containing papain (0.15 mg/ml) and bovine serum albumin (0.02%). After 17 h, dithiothreitol (0.1 mM) was added, and the preparation was gently agitated at 37°C for 20 min. The medial layers were then transferred to the culture medium (Ham's F10 and MEM, 1:1 v/v, supplemented with 2% Ultroser G, 0.05 mM ascorbic acid and 2 mM glutamine) and cells were dissociated by trituration. After preplating, used to obtain pure vascular smooth muscle cell cultures [14], the cell suspension was transferred into 24-well cluster trays (Costar) and placed at 37°C in a humidified incubator, gassed with 95% air/5% CO_2. When confluence was reached, the cultures were passaged once using 1.5 g/l trypsin. The medium was changed every 3 days.

2.3. Incubation of cells and nitrite determination

Confluent vascular smooth muscle cells were incubated for 24 h in MEM without L-arginine, in the absence or presence of OH-L-Arg (1–1000 μM), with L-NAME (300 μM), miconazole (100 μM) or LPS (10 mg/ml) alone or in various combinations, as indicated. Thereafter, nitrite concentrations were determined in the medium using Griess' reagent (1% sulfanilamide, 0.1% napthylethylenediamine, 2.5% orthophosphoric acid). Briefly, 100 ml of Griess' reagent was mixed with an equal volume of medium, and optical density was determined at 540 nm after 10 min.

2.4. Interaction of OH-L-arg and cationic amino acids with [^{3}H]L-arginine uptake

Interactions with L-arginine transport were studied in rat aortic smooth muscle cells cultured as indicated above. [^{3}H]L-Arginine uptake was assayed in cells cultured on 24-well cluster trays using the methods described by Gazzola et al. [15]. Briefly, confluent cells were washed and incubated in HBSS for 60 min at 37°C. The HBSS was then removed and the assay was initiated by the simultaneous addition to each well of 0.2 ml HBSS containing [^{3}H]L-arginine (at the indicated concentrations) and various concentrations of L-arginine, OH-L-Arg, L-lysine or L-ornithine. Experiments were performed at 37°C with an incubation time of 1 min. The assay was terminated by washing 3 times with ice-cold phosphate-buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 3.38 mM, KH_2PO_4 1.47 mM, pH 7.4). The incubation time was chosen in view of the results of preliminary experiments showing that [^{3}H]L-arginine uptake was linear for 3 min in these conditions. After extraction with 10% trichloroacetic acid, 200 μl aliquots were taken for determination of [^{3}H]L-arginine by scintillation counting.

2.5. Statistical analysis

Results are expressed as mean ± standard error mean (S.E.M.) of n experiments, n representing the number of wells studied. Statistical comparisons were made using ANOVA and Student's t-test as indicated. P values less than 0.05 were considered to be statistically significant. Concentrations giving half-maximal effects (EC_{50}) or 50% inhibition (IC_{50}) and slope factors were calculated by logit–log regression. The L-arginine uptake inhibition constant values (K_i) were calculated from IC_{50} values according to Cheng and Prussof [16], using a Michaelis constant (K_m) value of 105 μM for [^{3}H]L-arginine [11].

3. Results

3.1. Effect of OH-L-Arg on nitrite production

Incubation of aortic smooth muscle cells with OH-L-Arg induced a concentration dependent increase in nitrite accumulation in the culture medium (Fig. 1). The half-maximal effect was obtained with 18.0 ± 1.5 μM (n = 4–7) and maximal nitrite accumulation was obtained with 100 μM. This effect was not due to spontaneous oxidation, since in the absence of cells, no significant increase in nitrite was detected. Incubation of vascular smooth muscle cells with L-arginine (300 mM) was without effect on nitrite concentrations in the medium (1.83 ± 0.69 μM and 1.87 ± 0.1 μM, in the absence and presence of L-arginine, respectively; n = 3–5).

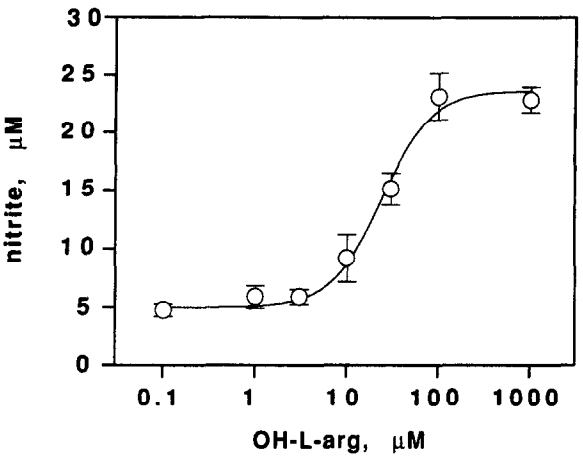

Fig. 1. Nitrite production by vascular smooth muscle cells incubated for 24 h in the presence of OH-L-Arg. Results are presented as mean ± S.E.M., n = 4–7.

As shown in Fig. 2, L-NAME (300 mM) was without significant effect on basal and OH-L-Arg induced nitrite production. For example, with 100 μM OH-L-Arg, nitrite concentrations were respectively 17.91 ± 0.41 and 17.25 ± 0.66 μM, in the absence and presence of L-NAME. These data are consistent with the absence of NO-synthase activity in rat aortic smooth muscle cells, under these experimental conditions. In contrast, miconazole (100 μM) an inhibitor of cytochrome P_{450}, reduced nitrite production elicited by OH-L-Arg at both concentrations used to basal levels (Fig. 2).

3.2. Effect of NO-synthase induction on OH-L-Arginine induced nitrite production

As shown in Fig. 3, incubation of smooth muscle cells with LPS induced a significant increase in nitrite concentration. This increase could be prevented by co-incubation with L-NAME (300 μM, data not shown), consistent with the induction of an inducible NO-synthase. In the presence of OH-L-Arg, LPS did not increase significantly nitrite accumulation compared to OH-L-Arg alone (Fig. 3). The results show that the effects of LPS and OH-L-Arg on nitrite production were not additive.

3.3. [^{3}H]L-Arginine uptake

The effect of OH-L-Arg on L-arginine uptake in rat aortic smooth muscle cells was compared with those of

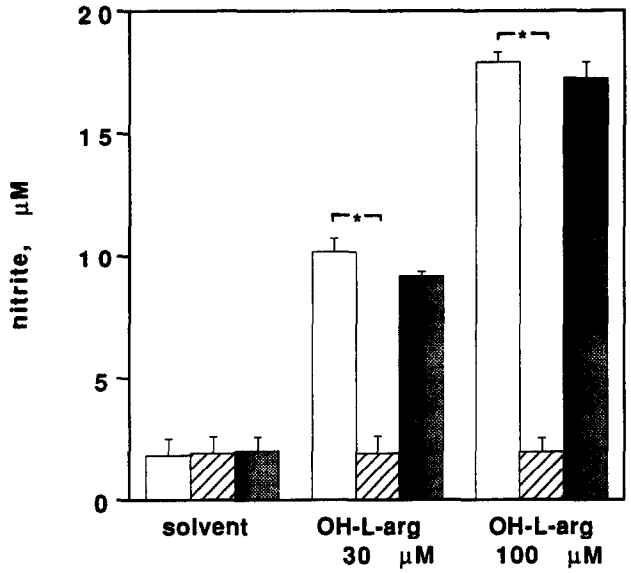

Fig. 2. Histograms depicting the effects of OH-L-Arg (30–100 μM) alone (open columns), or together with L-NAME (300 μM, closed columns) or miconazole (100 μM, hatched columns) on nitrite accumulation in rat aortic smooth muscle cell cultures. Results are presented as mean ± S.E.M., $n = 4$–8. Significant difference between groups: $^*P < 0.001$.

cationic amino acids which enter into cells through the y^+ transporter. As illustrated in Fig. 4, [^{3}H]L-arginine (100 μM) uptake was inhibited in a concentration-dependent manner by L-lysine, L-ornithine and OH-L-Arg. The K_i values calculated from the IC_{50} values of the curves illustrated in Fig. 4 were 260 ± 40 μM ($n = 5$) for L-lysine, 330 ± 10 mM ($n = 5$) for L-ornithine and 195 ± 23 mM ($n = 12$) for OH-L-Arg. In slightly different conditions (70 μM [^{3}H]L-arginine), the K_i for L-arginine was 118 ± 18 μM ($n = 4$, data not shown). Furthermore, the uptake could be totally abolished by these compounds. In all cases, the slope factor was not significantly different from unity, indicating that the interaction was competitive.

4. Discussion

The present results show that in vascular smooth muscle cells, exogenous OH-L-Arg is recognized by the L-arginine transporter and is oxidized into nitrite, independently of the induction of a NO-synthase.

In the absence of LPS, only a very slight nitrite production was detected, which was not inhibited by L-NAME. Thus, consistent with a previous report [8], rat aortic smooth muscle cells used here exhibited no significant NO-synthase activity. At the concentration used here (300 μM), L-NAME abolished LPS-induced nitrite formation (this study) and cyclic GMP accumulation [8] in rat aortic smooth muscle cells. The presently reported lack of effect of L-NAME on the conversion of OH-L-Arg into nitrite supports the view that a pathway not involving NO-synthase accounts for this conversion in smooth muscle cells. Such a pathway may also exist in endothelial cells, since it has been previously reported that L-NAME is also unable to abolish the endothelium-dependent relaxation induced by OH-L-Arg [17].

Contrary to L-NAME, miconazole, an inhibitor of cytochrome P_{450}, abolished nitrite production elicited by exposure to OH-L-Arg. This finding suggests that cytochrome P_{450} is able to catalyze the conversion of OH-L-Arg to NO in vascular smooth muscle cells. Such an oxidation of OH-L-Arg to citrulline and NO has been recently described in rat liver microsomes [18], in which a cytochrome P_{450} of the 3A subfamily is mainly responsible for the oxidation. In addition, based on the hypothetical reaction scheme proposed by Klatt et al. [3] for NO-synthase, the sequence of electron transfers and the involvement of a hydroxylating ferryl compound are in accord with established cytochrome P_{450} chemistry. Thus, cytochrome P_{450} is able to catalyze the second step of the conversion of L-arginine to L-citrulline and NO, through the intermediate formation of OH-L-Arg. Other oxidases are also able to catalyze this second step [19], but the inhibition by miconazole suggests that cytochrome P_{450} accounts for at least a large part of the conversion of OH-L-Arg to NO in rat aortic smooth muscle cells. Alternatively, a cytochrome P_{450} by-product such as hydrogen peroxide or superoxide might indirectly be involved in oxidation of OH-L-Arg by another enzyme.

The y^+ transport system for cationic amino acids has been characterized in many tissues [20], including rat aortic smooth muscle cells [10], where it accounts for

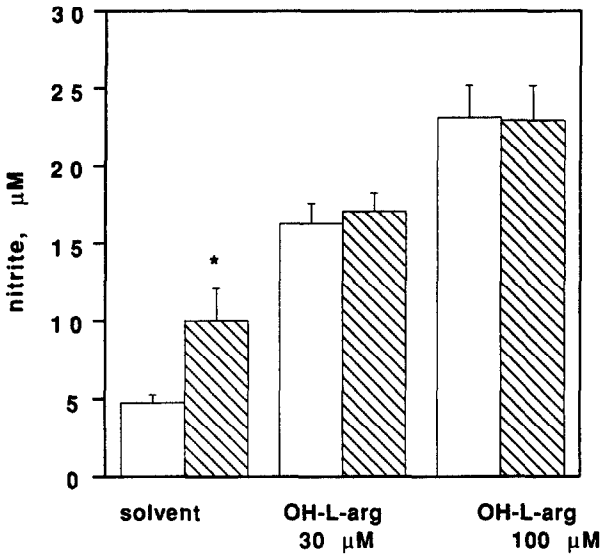

Fig. 3. Histograms showing the effect of solvent (open columns) and LPS (10 μg/ml, hatched columns) on nitrite accumulation in the medium of rat aortic smooth muscle cell cultures incubated with or without OH-L-Arg (0, 30 or 100 μM). Results are expressed as the mean ± S.E.M., $n = 4$–7. Significant difference between groups: $^*P < 0.001$.

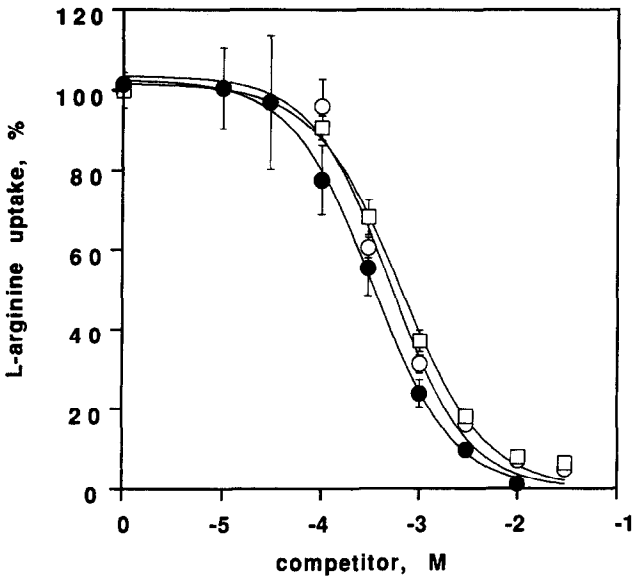

Fig. 4. Inhibition of [^{3}H]L-arginine (100 μM) uptake into rat aortic smooth muscle cells in the presence of various concentrations of L-lysine (○), L-ornithine (□) or OH-L-Arg (●). Mean ± S.E.M., n = 4–5.

L-arginine uptake [11]. Competitive inhibition of [^{3}H]L-arginine uptake by L-lysine, L-ornithine and OH-L-Arg and the corresponding K_i values found here, are consistent with OH-L-Arg interacting with this transporter with an affinity comparable to those of L-lysine and L-ornithine. It is therefore likely that OH-L-Arg enters into vascular smooth muscle cells via this transporter, as does L-arginine.

The K_i value of OH-L-Arg for the L-arginine transporter found here (195 μM) is about 10-fold higher than the concentration producing half-maximal nitrite production (18 μM). Such an apparent discrepancy between the K_m or K_i values of L-arginine for its transporter (about 100 μM) and the concentration producing half-maximal relaxation (8 μM) in rat aortic rings exposed to LPS, has also been described previously [11,21]. K_m values of L-arginine and OH-L-Arg are respectively about 100 and 200 μM for the transporter [11 and this study] and about 1 to 10 μM for NO-synthase [2,3]. Due to the low K_m of the enzyme compared to the transporter, the intracellular substrate concentration allowing maximal enzyme velocity may be reached in the presence of extracellular substrate concentrations far below those saturating the transporter, if the capacity of the transporter is sufficient. However, the K_m of OH-L-Arg for cytochrome P_{450} is still unknown. In addition, it cannot be ruled out that OH-L-Arg enters into rat aortic smooth muscle cells through another (unidentified) transporter, other than that for L-arginine, thus perhaps accounting in part for NO production at low OH-L-Arg concentrations.

The lack of additive effects of LPS and OH-L-Arg on nitrite formation is also consistent with the view that intracellular oxidase activity was sufficient to entirely convert OH-L-Arg, entering into cells into NO, even in the absence of NO-synthase. However, the mechanisms underlying the interactions of LPS and OH-L-Arg may be complex. For example, an inhibition of the inducible NO-synthase by NO produced from OH-L-Arg and cytochrome P_{450} might be involved. Such a regulation has been described for the constitutive brain NO-synthase [22]. In addition, OH-L-Arg may react with NO to form a more stable compound [17,23], thereby decreasing its degradation to nitrite. Thus, various mechanisms with opposing effects on nitrite formation may be responsible for the absence of additive effects of LPS and OH-L-Arg.

Taken together, the present results show that OH-L-Arg can enter into vascular smooth muscle cells, where it is oxidized into NO, independently of the presence of NO-synthase, and further degraded into nitrite. These findings suggest that OH-L-Arg released from some cells may exert paracrine actions on vascular smooth muscle cells, especially when it is formed in large quantities. This might be the case in endotoxemia, during which a large increase in NO production in blood vessels accounts for cyclic GMP accumulation and hyporeactivity to vasoconstrictor agents [8,24].

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