Inhibition of arginase by N^G-hydroxy-L-arginine in alveolar macrophages: implications for the utilization of L-arginine for nitric oxide synthesis

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Abstract The hypothesis was investigated that the nitric oxide (NO) synthase intermediate, N^G-hydroxy-L-arginine (HOArg), is an arginase inhibitor in rabbit or rat alveolar macrophages. Exogenously applied HOArg strongly inhibited the arginase activity present in these cells ($IC_{50} \geq 15$ μM), and attenuated L-[^{3}H]arginine transport ($IC_{50} \geq 500$ μM) in rabbit alveolar macrophages. Moreover, up to 37 μM HOArg were detected in the conditioned medium, but not in the lysate, of rat alveolar macrophages exposed to bacterial lipopolysaccharide for 18 h. HOArg may thus be a potent endogenous arginase inhibitor in these cells which increases the availability of L-arginine for NO biosynthesis.

Key words: NO synthase; Arginase; Hydroxyarginine; Arginine; Macrophage; Lipopolysaccharide; *E. coli*

1. Introduction

Following exposure to bacterial lipopolysaccharide (LPS) and/or various cytokines, macrophages express the Ca^{2+}-independent isoform of nitric oxide (NO) synthase [1]. These cells also contain substantial amounts of arginase, the expression of which may also be upregulated in response to LPS [2]. Provided that the K_m values and total activities of NO synthase and arginase are comparable, the two enzymes may compete for the endogenous L-arginine, the availability of which would eventually become rate-limiting for the synthesis of NO or L-ornithine.

Recently, the NO synthase intermediate [3], N^G-hydroxy-L-arginine (HOArg), has been shown to be a potent inhibitor of liver arginase [4,5]. Since substantial amounts of this intermediate appear to be liberated from the active site of NO synthase [6,7], it is conceivable that HOArg may act as an endogenous arginase inhibitor in NO-producing cells, such as macrophages.

We have investigated this hypothesis by comparing the endogenous formation of HOArg in LPS-stimulated alveolar macrophages with the effects of this amino acid on the arginase activity present in these cells.

2. Materials and methods

2.1. Materials

N^G-Hydroxy-L-arginine (HOArg) was generously supplied by Glaxo Group Research Ltd. L-[2,3,4,5-^{3}H]Arginine (2.0–2.2 TBq/mmol) and L-[U-^{14}C]ornithine (10 GBq/mmol) were obtained from Amersham; L-[U-^{14}C]citrulline (2.2 GBq/mmol) from NEN-DuPont; N^G-monomethyl-L-arginine, mouse interferon-γ (IIFN-γ) and LPS (serotype 0127:B08) from Sigma; and N^G-nitro-L-arginine from Serva.

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2.2. Cell culture and experimental protocols

Alveolar macrophages from Sprague–Dawley rats (200–250 g) or mongrel rabbits (1.5–2.0 kg) of either sex were prepared by bronchoalveolar lavage essentially as previously described [8]. Cells (2.5×10^6/well) were seeded into 6-well plates containing DMEM/Ham's F-12 medium (ICN Biomedicals) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml amphotericin B and 5% fetal calf serum (FCS; Vitromex). Non-adherent cells were removed by exchanging the medium 4 times at 1 h intervals. Adherent cells consisting of ≥90% macrophages (May–Grünwald–Giemsa staining) with a viability of ≥95% (Trypan blue exclusion) were cultured for 18 h in the absence or presence of LPS (10 μg/ml). The conditioned medium was removed and stored at −70°C prior to analysis by HPLC for the amino acid pattern and colourimetric determination of the concentration of nitrite (NO_2^-) [8]. In some experiments, the cells were lysed by adding 4 vols. of ice-cold methanol for HPLC analysis of the intracellular amino acid content.

In another series of experiments the cells were washed and incubated for 60 min at 37°C in 1 ml HEPES-modified Krebs solution, pH 7.4 (composition in mM: NaCl 118.5, KCl 5.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, NaH_2PO_4 0.001, Na_2EDTA 0.03, (+)ascorbic acid 0.06, HEPES 20.0, D-glucose 11.1), containing 37 kBq L-[^{3}H]arginine (~20 μM). The supernatants were removed and stored at −70°C prior to HPLC analysis.

Arginine uptake by rabbit alveolar macrophages was measured after resuspension of 10^6 freshly isolated cells in 0.5 ml Krebs solution containing L-[^{3}H]arginine (37 kBq, ~20 μM). Incubations were terminated after 5 min by adding 1 ml ice-cold Krebs solution. The cells were washed, collected by centrifugation, lysed with 0.4 M $HClO_4$ (0.5 ml), and the cell-associated radioactivity determined by using a liquid scintillation β-counter. Values for non-specific uptake at 4°C were subtracted from those obtained at 37°C to calculate specific L-[^{3}H]arginine uptake.

2.3. HPLC analyses

HPLC/fluorescence analysis of the concentration of L-citrulline, L-arginine and HOArg in the supernatant or lysate of LPS-stimulated macrophages was performed by pre-column derivatization with *o*-phthalaldehyde as previously described [10], except that the column (250 × 4.6 mm (i.d.) Ultra Techsphere ODS, HPLC Technology) was isocratically eluted with 10 mM KH_2PO_4, pH 5.85/acetonitrile/methanol/tetrahydrofuran 79:10:10:1 (v/v/v/v) at a flow rate of 1 ml/min. L-Arginine, L-citrulline and HOArg were eluted from the column with retention times of 20.5, 14.1 and 18.5 min, respectively.

HPLC analysis of the concentration of radio-labelled L-arginine, L-citrulline and L-ornithine was performed by using a 250 × 4.6 mm (i.d.) Hypersil ODS column (Shadon) which was isocratically eluted with 0.1 M NaH_2PO_4, pH 1.8 (containing 400 mg/l octane sulfonic acid and 0.3 mM Na_2EDTA and 6.25% (v/v) methanol) at a flow rate of 1 ml/min. The radioactivity of the eluate (1 ml fractions) was monitored by using a liquid scintillation β-counter, and the retention times of the amino acids determined by comparison with those of authentic L-[^{3}H]arginine (60 min), L-[U-^{14}C]ornithine (16 min) and L-[U-^{14}C]citrulline (8 min).

2.4. Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means ± S.E.M. of *n* observations. Statistical evaluation was performed by one way analysis of variance followed by a Bonferroni *t*-test for multiple comparisons with a P value < 0.05 considered statistically significant.

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3. Results

3.1. HOArg synthesis by LPS-stimulated macrophages

Exposure of rat alveolar macrophages to LPS for 18 h resulted in a ~12-fold increase in the concentration of NO_2^- and L-citrulline in the conditioned medium (Fig. 1) which was paralleled by a comparable decrease in the level of L-arginine (to 12% of the level in the supernatant of control cells). HOArg levels were also markedly elevated (to 7.5–36.9 μM, average 21.1 ± 4.8 μM), the concentration of which accounted for 6.1 ± 0.9% of the L-arginine consumed by the macrophage NO synthase (n = 16). Interestingly, HOArg was not detectable in lysates of LPS-treated rat alveolar macrophages, whereas both the intracellular and extracellular concentration of L-citrulline were elevated to the same extent (not shown). Treatment of the cells with the NO synthase inhibitor, N^G-monomethyl-L-arginine (100 μM), significantly attenuated the LPS-induced increase in the concentration of NO_2^- and L-citrulline (by 66% and 82%, respectively), but did not reduce the level of HOArg (Fig. 1). NO synthase inhibition also restored the level of L-arginine to 79% of the concentration in the supernatant of control cells.

LPS-treatment of rabbit alveolar macrophages did not lead to a substantial increase in inducible NO synthase activity, since the level of NO_2^-, L-citrulline and HOArg (Fig. 1, insert) in the conditioned medium was increased only by ~50% as compared to the supernatant of control cells. Co-incubation with N^G-nitro-L-arginine (100 μM), another NO synthase inhibitor, completely prevented the increase in HOArg.

3.2. Arginase inhibition by HOArg

Rabbit alveolar macrophages exposed to L-[^{3}H]arginine for 1 h produced significant amounts of L-[^{3}H]ornithine. Moreover,

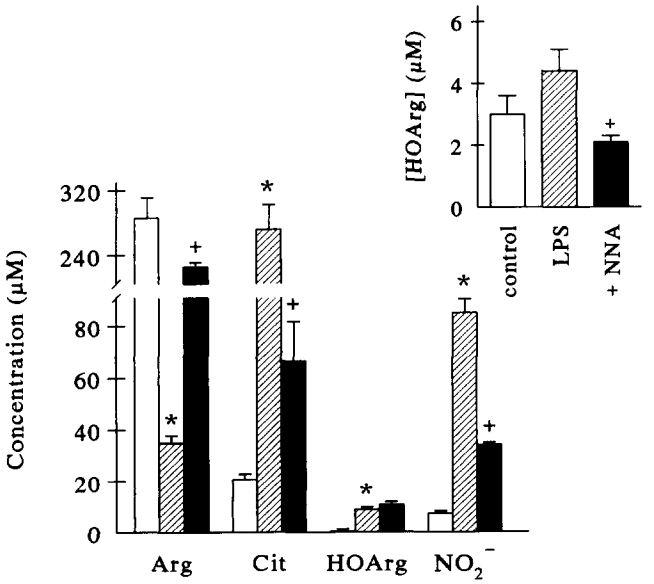

Fig. 1. Generation of HOArg by LPS-stimulated alveolar macrophages. Rat or rabbit (insert) alveolar macrophages were incubated in culture medium in the absence (open columns) or presence (hatched columns) of LPS for 18 h, with or without 100 μM N^G-monomethyl-L-arginine (filled columns) or N^G-nitro-L-arginine (+ NNA). Thereafter the concentration of nitrite (NO_2^-), L-arginine (Arg), L-citrulline (Cit) and HOArg was determined in the conditioned medium (n = 3–4; $^*P < 0.05$ vs. control, $^+P < 0.05$ vs. LPS).

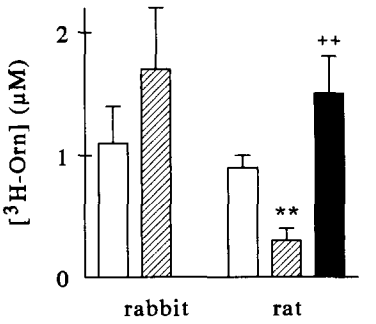

Fig. 2. Effects of LPS on arginase activity. Rabbit or rat alveolar macrophages were cultured for 18 h in the absence (open columns, n = 13–24) or presence (hatched columns, n = 13–37) of LPS, with or without N^G-monomethyl-L-arginine (100 μM, n = 13, filled columns). Thereafter, the cells were incubated with 20 μM L-[^{3}H]arginine for 1 h, and the concentration of L-[^{3}H]ornithine in the supernatant (^{3}H-Orn) was determined by HPLC analysis ($^{**}P < 0.01$ vs. control, $^{++}P < 0.01$ vs. LPS).

L-[^{3}H]ornithine formation was ~60% higher in LPS-treated cells (Fig. 2). Unstimulated rat alveolar macrophages generated similar amounts of L-[^{3}H]ornithine. In contrast to rabbit alveolar macrophages, L-[^{3}H]ornithine formation was significantly reduced after LPS-treatment ($P < 0.01$). Co-treatment of rat alveolar macrophages with N^G-monomethyl-L-arginine, on the other hand, resulted in an increase rather than decrease in L-[^{3}H]ornithine formation (Fig. 2).

Exogenously applied HOArg inhibited the formation of L-[^{3}H]ornithine from L-[^{3}H]arginine by LPS-treated rabbit alveolar macrophages (Fig. 3a) with a half-maximally effective concentration (IC_{50}) of 15 μM. HOArg also reduced the arginase activity present in unstimulated rat alveolar macrophages in a concentration-dependent manner with an IC_{50} of 41 μM (Fig. 3b). Interestingly, HOArg also inhibited the uptake of L-[^{3}H]arginine by unstimulated rabbit alveolar macrophages (Fig. 3c). However, the half-maximally effective concentration (500 μM) for the inhibition of L-[^{3}H]arginine uptake was much higher than that required for the inhibition of arginase.

4. Discussion

The present findings demonstrate that induction of NO synthase in LPS-stimulated rat alveolar macrophages not only causes an increased production of NO (measured as NO_2^-) and L-citrulline, but also markedly enhances the release of the NO synthase intermediate, HOArg, from these cells. The peak concentration of HOArg in the conditioned medium was 37 μM. At this concentration, arginase activity in these cells was reduced by ≥ 50%. Moreover, L-[^{3}H]ornithine formation by rabbit alveolar macrophages was already significantly affected at concentrations as low as 1 μM, supporting the notion that HOArg is the most potent arginase inhibitor known to date [4,5].

The hypothesis that HOArg may be an endogenous arginase inhibitor in alveolar macrophages is supported by the N^G-monomethyl-L-arginine-sensitive decrease in arginase activity in LPS-stimulated rat alveolar macrophages as compared to LPS-stimulated rabbit alveolar macrophages, in which NO synthase activity (i.e. HOArg formation) was only marginally elevated. The finding that the formation of L-[^{3}H]ornithine by

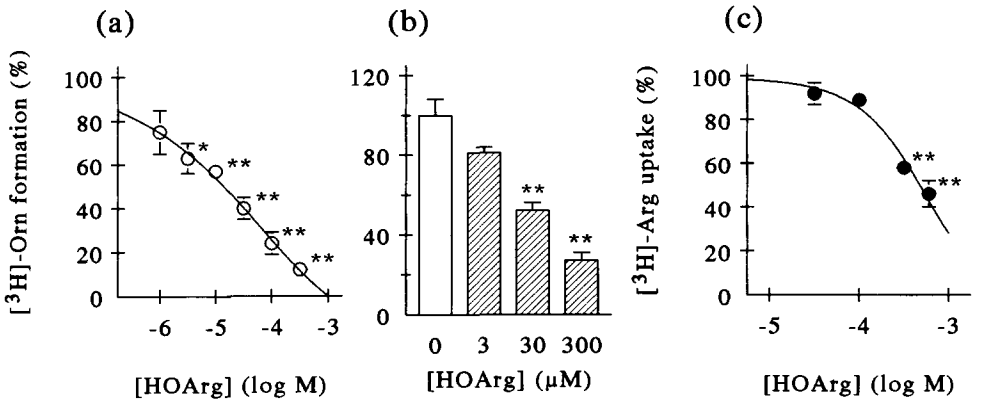

Fig. 3. Effects of HOArg on arginase activity (a,b) and on L-arginine transport (c). LPS-stimulated rabbit (a) or unstimulated rat (b) alveolar macrophages were incubated with L-[^{3}H]arginine for 1 h in the absence or presence of HOArg. L-[^{3}H]Ornithine ([^{3}H]-Orn) formation was determined by HPLC analysis and expressed as percentage of the mean control values. (c) Unstimulated rabbit alveolar macrophages were incubated with L-[^{3}H]arginine ([^{3}H]-Arg) for 5 min, and thereafter the cell-associated radioactivity was determined by liquid scintillation β-counting (n = 3–8; $^*P < 0.01$, $^{**}P < 0.01$ vs. control, i.e. 100%).

LPS-stimulated rat alveolar macrophages was actually increased in the presence of the NO synthase inhibitor as compared to control cells may reflect an enhanced expression of arginase [2].

In contrast to its potent effects on arginase activity, L-[^{3}H]arginine transport in rabbit alveolar macrophages was inhibited by HOArg only at concentrations $\geq 100\ \mu$M (IC_{50} 500 μM). Similar findings (K_i 195 μM) have recently been reported for L-[^{3}H]arginine transport in vascular smooth muscle cells [11] which like that in macrophages appears to be mediated by the y^+ carrier system. A competition between L-arginine and HOArg for the same amino acid carrier could indeed explain why HOArg accumulates in the conditioned medium of LPS-treated alveolar macrophages. Moreover, the fact that no HOArg was detectable in cell lysates (cf. [6]) may indicate that any HOArg remaining in the cell is eventually metabolized to NO and L-citrulline either by NO synthase itself or other enzymes, such as cytochrome P-450 monooxygenases [11,12], or is perhaps bound to arginase. On the basis of the present experiments, however, we cannot rule out a transport of HOArg via a neutral amino acid carrier system.

The notion that a decrease in the extracellular level of L-arginine below a critical threshold may facilitate HOArg reuptake and metabolism, on the other hand, is supported by the linear correlation (r = 0.987) of the decrease in the concentration of both HOArg and L-arginine in the conditioned medium of LPS-stimulated rat alveolar macrophages (n = 10, $P < 0.01$). A competition between HOArg and L-arginine for the same amino acid carrier may also be the reason for the apparent lack of effect of N^G-monomethyl-L-arginine on the level of HOArg in the conditioned medium of LPS-stimulated rat alveolar macrophages. As shown in Fig. 1, the NO synthase inhibitor largely prevented the marked decrease in the extracellular concentration of L-arginine following LPS treatment but, as judged by the level of NO_2^- and L-citrulline, left ~25% of the NO synthase activity intact. When considering the capacity of rat alveolar macrophages to generate HOArg (up to 37 μM) and the fact that NO synthase is the only known source of HOArg, it is very likely that in this series of experiments the actual amount of HOArg produced by the cells following exposure to LPS has been underestimated on the basis of the extracellular HOArg level. Indeed, exposure of cultured vascular smooth muscle cells to interleukin-1β or RAW 264.7 mouse macrophages to LPS also results in a significant production of HOArg which is abolished in the presence of N^G-nitro-L-arginine. The enhanced formation of HOArg (plus NO_2^- and L-citrulline) by these cells, however, is accompanied by a less pronounced decrease in the concentration of L-arginine in the conditioned medium (not below 130 μM) than that seen with the LPS-stimulated rat alveolar macrophages (Hecker et al., unpublished observations).

It is remarkable that up to 11% of the L-arginine consumed by the NO synthase in LPS-stimulated rat alveolar macrophages appeared as HOArg in the extracellular space. An accumulation of considerable amounts of HOArg ($\geq$17%) has also been described in the conditioned medium of LPS/IFN-γ-stimulated murine mammary adenocarcioma cells (EMT-6, [6]), and small amounts of HOArg ($\leq$2%) were detected upon incubation of the purified neuronal NO synthase with L-arginine [7]. These findings suggest that a significant portion of the HOArg which is generated in the course of the NO synthase reaction can be liberated from the active site of the enzyme and may thus escape further metabolism to NO and L-citrulline. This may be a consequence of the twofold higher K_m value of the NO synthase for HOArg as compared to L-arginine [3,7] which favours its displacement from the active site by L-arginine.

In summary, the present findings demonstrate that LPS-stimulated murine macrophages release substantial amounts of HOArg which can affect arginase activity. Since exposure of macrophages to LPS may lead to a co-induction of NO synthase and arginase [2], the increased level of HOArg could limit arginase activity and direct L-arginine utilization towards an enhanced synthesis of NO. Moreover, by reducing the availability of L-ornithine for polyamine synthesis, macrophage-derived HOArg may exert cytostatic effects, e.g. on tumor cells [6]. It remains to be determined, however, as to how HOArg leaves the cell which produces it.

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