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Inhibition of arginase in rat and rabbit alveolar macrophages by N^{ω}-hydroxy-D,L-indospicine, effects on L-arginine utilization by nitric oxide synthase

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1 Alveolar macrophages (AMΦ) exhibit arginase activity and may, in addition, express an inducible form of nitric oxide (NO) synthase (iNOS). Both pathways may compete for the substrate, L-arginine. The present study tested whether two recently described potent inhibitors of liver arginase (N^{ω}-hydroxy-D,L-indospicine and 4-hydroxyamidino-D,L-phenylalanine) might also inhibit arginase in AMΦ and whether inhibition of arginase might affect L-arginine utilization by iNOS.

2 AMΦ obtained by broncho-alveolar lavage of rat and rabbit isolated lungs were disseminated (2.5 or 3×10^6 cells per well) and allowed to adhere for 2 h. Thereafter, they were either used to study [^{3}H]-L-arginine uptake (37 kBq, 0.1 μM, 2 min) or cultured for 20 h in the absence or presence of bacterial lipopolysaccharide (LPS). Cultured AMΦ were incubated for 1 h with [^{3}H]-L-arginine (37 kBq, 0.1 μM) and the accumulation of [^{3}H]-L-citrulline (NOS activity) and [^{3}H]-L-ornithine (arginase activity) was determined.

3 During 1 h incubation of rabbit AMΦ with [^{3}H]-L-arginine, no [^{3}H]-L-citrulline, but significant amounts of [^{3}H]-L-ornithine (150 d.p.m. $\times$ 1000) were formed. N^{ω}-hydroxy-D,L-indospicine and 4-hydroxyamidino-D,L-phenylalanine, present during incubation, concentration-dependently reduced [^{3}H]-L-ornithine formation (IC_{50}: 2 and 45 μM, respectively).

4 N^{ω}-hydroxy-D,L-indospicine (up to 100 μM) had no effect on [^{3}H]-L-arginine uptake into rabbit AMΦ, whereas 4-hydroxyamidino-D,L-phenylalanine caused a concentration-dependent inhibition (IC_{50}: 300 μM).

5 Rat AMΦ, cultured in the absence of LPS, formed significant amounts of [^{3}H]-L-citrulline and [^{3}H]-L-ornithine (133 and 212 d.p.m. $\times$ 1000, respectively) when incubated for 1 h with [^{3}H]-L-arginine. When AMΦ had been cultured in the presence of 0.1 or 1 μg ml^{-1} LPS, the formation of [^{3}H]-L-citrulline was enhanced by 37 ± 8.3 and 99 ± 12% and that of [^{3}H]-L-ornithine reduced by 21 ± 8.7 and 70 ± 2.5%, respectively.

6 In rat AMΦ, cultured in the absence or presence of LPS, N^{ω}-hydroxy-D,L-indospicine (10 and 30 μM) greatly reduced formation of [^{3}H]-L-ornithine (by 80–95%) and this was accompanied by increased formation of [^{3}H]-L-citrulline. However, only 20–30% of the [^{3}H]-L-arginine not metabolized to [^{3}H]-L-ornithine after inhibition of arginase was metabolized to [^{3}H]-L-citrulline, when the AMΦ had been cultured in the absence of LPS (i.e. low level of iNOS). On the other hand, when the AMΦ had been cultured in the presence of LPS (i.e. high level of iNOS), all the [^{3}H]-L-arginine not metabolized by the inhibited arginase was metabolized to [^{3}H]-L-citrulline.

7 In conclusion, N^{ω}-hydroxy-D,L-indospicine is a potent and specific inhibitor of arginase in AMΦ. In cells in which, in addition to arginase, iNOS is expressed, inhibition of arginase can cause a shift of L-arginine metabolism to the NOS pathway. However, the extent of this shift appears to depend in a complex manner on the level of iNOS.

Keywords: Alveolar macrophages; airway inflammation; nitric oxide; nitric oxide synthase; arginase; L-arginine metabolism; N^{ω}-hydroxy-D,L-indospicine

Introduction

In alveolar macrophages (AMΦ), as in other cells of the monocyte/macrophage lineage, L-arginine may be the substrate of different enzyme pathways. Arginase, which catalyses the hydrolysis of L-arginine to L-ornithine, a precursor of polyamine synthesis, appears to be expressed constitutively in different types of macrophages (MΦ) including AMΦ (e.g. Hoffman *et al*., 1978; Curie, 1978), although different stimuli may modulate the level of expression of this enzyme (e.g. Corraliza *et al*., 1995; Hey *et al*., 1995; Modolell *et al*., 1995; Nematholahi *et al*., 1995; Wang *et al*., 1995). In MΦ and AMΦ an inducible form of nitric oxide (NO) synthase (iNOS) may also be expressed (e.g. Yui *et al*., 1991; Jorens *et al*., 1991; Nozaki *et al*., 1993; Hey *et al*., 1995; for reviews see Moncada *et al*., 1991; Dugas *et al*., 1995). However, it appears that there are marked species differences with regard to the mechanisms controlling the induction of iNOS in MΦ and AMΦ. Whereas in MΦ or AMΦ of rats or mice iNOS can easily be induced, in other species including man the induction of iNOS appears to be much more complex (e.g. Hey *et al*., 1995; for reviews see Denis, 1994; Dugas *et al*., 1995).

Since arginase and NOS use L-arginine as a common substrate, competition between both enzymes for the substrate may occur when iNOS is induced. Indeed, induction of iNOS in rat AMΦ was accompanied by a reduction of L-arginine

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utilization by arginase (Hey *et al*., 1995). Since inhibition of NOS restored the ornithine formation (Hey *et al*., 1995), a reduction of the amount of available arginase may not have been responsible for the apparent reduction in arginase activity. Evidence was obtained that induction of iNOS not only lowers the substrate availability for arginase, but in addition may cause inhibition of arginase by the liberation of N^{ω}-hydroxy-L-arginine.

N^{ω}-hydroxy-L-arginine, an intermediate in the NOS pathway, is a potent inhibitor of arginase in the liver (Daghigh *et al*., 1994; Boucher *et al*., 1994), murine peritoneal MΦ (Boucher *et al*., 1994) and rat and rabbit AMΦ (Hecker *et al*., 1995). Moreover, after induction of iNOS in rat AMΦ, N^{ω}-hydroxy-L-arginine was liberated in amounts sufficient to cause significant inhibition of arginase (Hecker *et al*., 1995).

On the other hand, it is not yet known whether changes in activity of arginase might affect NO synthesis. Until recently, potent inhibitors of arginase were not available, with the exception of N^{ω}-hydroxyl-L-arginine (Daghigh *et al*., 1994; Hecker *et al*., 1995). However, N^{ω}-hydroxy-L-arginine could not be used as a pharmacological tool to elucidate interactions between the arginase and NOS pathways, because N^{ω}-hydroxy-L-arginine itself is a substrate for NOS and thus may enhance the substrate supply to NOS. Very recently, N^{ω}-hydroxy-D,L-indospicine and 4-hydroxyamidino-D,L-phenylalanine have been described as potent and selective inhibitors of liver arginase with little effect on NOS (Vadon *et al*., 1996; Custot *et al*., 1996). Therefore, the aims of the present study were firstly to test whether these drugs might also inhibit arginase in AMΦ and then to test whether inhibition of arginase might promote utilization of L-arginine by NOS under conditions in which different levels of iNOS were induced. The present experiments were carried out in rabbits and rat AMΦ, because rabbit AMΦ, cultured in the absence or presence of bacterial lipopolysaccharide (LPS) do not express iNOS (Hey *et al*., 1995), whereas in rat AMΦ different levels of iNOS may easily be induced by culturing the cells in the presence of different concentrations of LPS (Jorens *et al*., 1991; Nozaki *et al*., 1993; Hey *et al*., 1995).

A preliminary account of part of the present study has been published (Hey *et al*., 1996a).

Methods

Preparation and incubation of AMΦ

Mongrel rabbits (2–2.5 kg, Lammers, Euskirchen) and Sprague Dawley rats (200–250 g) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised *en bloc*, washed with calcium and magnesium-free Dulbecco's phosphate buffered saline (D-PBS) and lavaged three times by instilling 60 ml (rabbit lung) or 15 ml (rat lung) of cold (4°C) D-PBS (see Holt, 1979; Hey *et al*., 1995). Usually for one preparation of AMΦ, lavage fluid from 1–2 rabbit lungs and 6–7 rat lungs were pooled and centrifuged at room temperature at 400 *g* for 5 min. The cells were washed three times with 2 ml D-PBS and thereafter resuspended in DMEM/F-12 medium supplemented with 5% v/v foetal calf serum, 2 mM glutamine, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 μg ml^{-1} amphothericin B and plated in sterile 6-well dishes (NUNC, Wiesbaden, Germany; 2.5×10^6 (turnover studies) or 3×10^6 (uptake studies) AMΦ per well). The AMΦ were allowed to adhere for 2 h (37°C, 5% CO_2). After 2 h the medium was renewed to remove non-adherent cells. The adherent cells consisted of more than 90% AMΦ according to morphological criteria (May Grünwald-Giemsa staining as described by Rick (1974)). In most experiments cells were cultured for 20 h in the absence (rabbit) or presence (rat) of LPS at the concentrations indicated. Cell viability was assessed by trypan blue exclusion and was always greater than 95%.

After this culture period the cells were washed and thereafter incubated at 37°C for 1 h in 1 ml Krebs-HEPES medium of the following composition (mM): NaH_2PO_4 0.001, NaCl 118.5, KCl 5.57, $CaCl_2$ 1.25, $MgCl_2$ 1.2, Na_2EDTA 0.03, (+)-ascorbic acid 0.06, HEPES 20.0 (adjusted to pH 7.4 with NaOH) and D-glucose 11.1. The medium contained in addition 37 kBq [^{3}H]-L-arginine (0.1 μM). The incubation media were collected and kept at −80°C until assayed by high performance liquid chromatography (h.p.l.c.) (see below).

L-Arginine uptake was measured immediately after the adherence period of the AMΦ. Adherent AMΦ were washed and incubated at 37°C for 2 min in 1 ml Krebs-HEPES medium containing [^{3}H]-L-arginine (37 kBq, 0.1 μM). Incubation was terminated by placing the plates on ice and rinsing AMΦ three times with 2 ml ice cold Krebs-HEPES medium containing 10 mM unlabelled L-arginine. Finally, AMΦ were extracted in 1 ml 0.4 M $HClO_4$ and radioactivity in the cell extracts were determined (see below). The 2 min incubation period was chosen as [^{3}H]-L-arginine accumulation was linear between 1 and 3 min, but showed rapid saturation between 5 and 10 min (data not shown). [^{3}H]-L-arginine uptake was expressed as d.p.m. per 3×10^6 AMΦ or as % of the uptake observed in controls of the respective cell preparation.

H.p.l.c. analysis

^{3}H compounds ([^{3}H]-L-citrulline, [^{3}H]-L-ornithine and [^{3}H]-L-arginine) in incubation media were separated as described previously (Hey *et al*., 1995) on a reverse phase column (length 250 mm, inner diameter 4.6 mm, prepacked with Shadon ODS-Hypersil, 5 μm) using as mobile phase a 0.1 M sodium phosphate buffer (adjusted to pH 1.8) which contained octane sulphonic acid sodium salt (400 mg l^{-1}), Na_2EDTA (0.3 mM) and methanol (6.25% v v^{-1}) with a flow rate of 1 ml min^{-1}. The h.p.l.c. eluate was collected in 1 min fractions into counting vials. The retention time of amino acids was determined by the use of ^{14}C-labelled (L-citrulline and L-ornithine) or ^{3}H-labelled (L-arginine) standards. L-Citrulline eluted after 7–8 min, ornithine after 12–13 min. L-Arginine eluted after about 80 min. Usually, the h.p.l.c. eluate of the first 25 min only was collected and the buffer eluting between 25 and 150 min was discarded. The amounts of [^{3}H]-L-citrulline and [^{3}H]-L-ornithine in the media were expressed as d.p.m. per well (i.e. 2.5×10^6 AMΦ) or as % of the mean value observed in the controls of the respective cell preparation.

Determination of radioactivity

Radioactivity in cell extracts and h.p.l.c. eluates was determined after addition of a commercial scintillation cocktail (Luma Safe, Canberra Packard, Frankfurt, Germany) by liquid scintillation spectrometry in a Beckman LS 5000TD (Beckman Instruments, Fullerton, U.S.A.). External standardization was used to correct for counting efficiency.

Statistical analysis

All values are mean ± s.e.mean of n experiments. Statistical significance of differences was evaluated by Student's t test. When multiple comparisons were performed the significance of differences was evaluated by the modified t test according to Dunnett by use of the computer programme GraphPad InStat (GraphPad Software, San Diego, U.S.A.). $P < 0.05$ was accepted as significant.

Drugs and materials

Amphotericin B (Sigma, München, Germany); L-[2,3-^{3}H]-arginine HCl (36.8 Ci $mmol^{-1}$, Sigma); Dulbecco's phosphate buffered saline without calcium and magnesium (D-PBS, Sigma); Dulbecco's modification of Eagle's medium/Ham's F-12 medium (DMEM/F-12 medium, Sigma); foetal calf serum (Vitromex, Germany); lipopolysaccharides from *Escherichia coli* 0127:B8 (LPS, Sigma). 4-Hydroxyamidino-D,L-phenylalanine and N^{ω}-hydroxy-D,L-indospicine were synthesized as

previously described (Vadon *et al*., 1996). Stock solutions of hydroxyamidino-D,L-phenylalanine and N^{ω}-D,L-indospicine (10 mM) and of LPS (500 μg ml^{-1}) were prepared in Dulbecco's phosphate buffered saline.

Results

Rabbit AMΦ

In confirmation of previous observations (Hey *et al*., 1995), no significant [^{3}H]-L-citrulline could be detected in media in which rabbit AMΦ (2.5×10^6 cells) had been incubated for 1 h with [^{3}H]-L-arginine (37 kBq, 0.1 μM). However, substantial amounts of [^{3}H]-L-ornithine accumulated in the media (150 ± 16 d.p.m. $\times 1000$, $n = 38$), corresponding to 7.0 ± 0.8% of the added [^{3}H]-L-arginine. N^{ω}-hydroxy-D,L-indospicine, present 30 min before and during incubation with [^{3}H]-L-arginine caused a concentration-dependent reduction in the formation of [^{3}H]-L-ornithine (Figure 1) without effects on the [^{3}H]-L-citrulline fraction (i.e. [^{3}H]-L-citrulline remained below the detection limit, data not shown). The concentration causing 50% reduction in [^{3}H]-L-ornithine formation (EC_{50}) was 2 μM. 4-Hydroxyamidino-D,L-phenylalanine also caused a concentration-dependent inhibition of [^{3}H]-L-ornithine formation by rabbit AMΦ, but with a lower potency (EC_{50}: 45 μM) (Figure 1).

The ability of these arginase inhibitors to affect [^{3}H]-L-arginine uptake into AMΦ was also tested. 4-Hydroxyamidino-D,L-phenylalanine produced a concentration-dependent inhibition of [^{3}H]-L-arginine uptake with a potency (EC_{50}: 300 μM) somewhat lower than that observed for inhibition of [^{3}H]-L-ornithine formation (Figure 2). On the other hand, N^{ω}-hydroxy-D,L-indospicine, up to a concentration of 100 μM, did not cause significant inhibition of [^{3}H]-L-arginine uptake (Figure 2). Thus, N^{ω}-hydroxy-D,L-indospicine appears to be the more selective arginase inhibitor and was therefore used in the following experiments.

Rat AMΦ

In contrast to rabbit AMΦ, iNOS can be easily induced in rat AMΦ (Hey *et al*., 1995). Thus, the effect of arginase inhibition

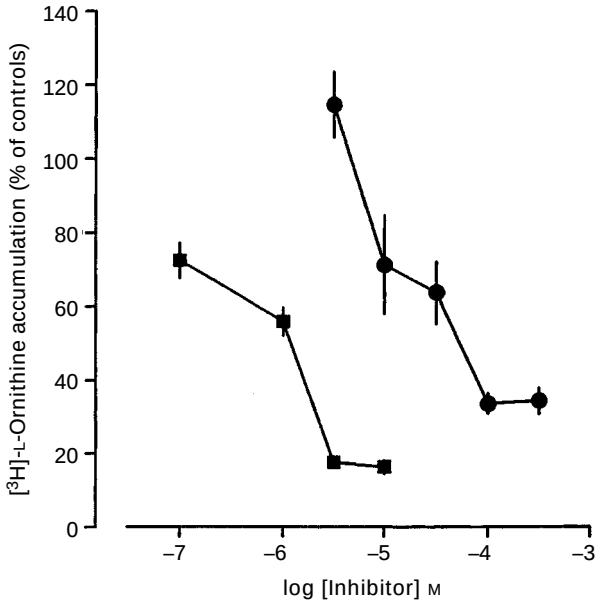

Figure 1 Concentration-dependent effects of N^{ω}-D,L-indospicine (■) and 4-hydroxyamidino-D,L-phenylalanine (●) on arginase activity in rabbit isolated AMΦ as determined by extracellular accumulation of [^{3}H]-L-ornithine during incubation with [^{3}H]-L-arginine. AMΦ were obtained by bronchoalveolar lavage of rabbit isolated lungs and disseminated in culture dishes (2.5×10^6 cells per well). They were cultured for 20 h. Thereafter, AMΦ were incubated for 1 h in 1 ml Krebs-HEPES solution containing [^{3}H]-L-arginine (37 kBq, 0.1 μM). Test drugs, at the concentrations indicated, were present 30 min before and during the incubation period with [^{3}H]-L-arginine. Ordinate scale: [^{3}H]-L-ornithine accumulation expressed as % of the respective mean value observed in controls of the individual cell preparation. Results show mean of 5–7 experiments; vertical lines indicate s.e.mean.

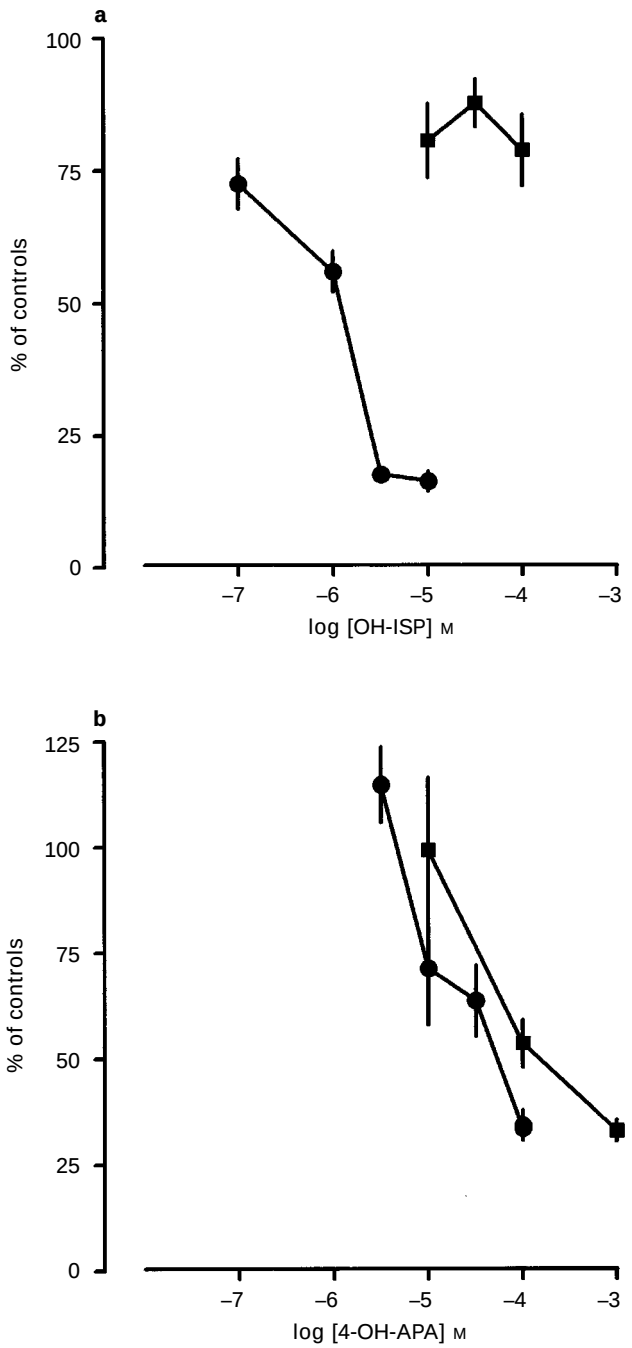

Figure 2 Comparison of the effects of (a) N^{ω}-hydroxy-D,L-indospicine (OH-ISP) and (b) 4-hydroxyamidino-D,L-phenylalanine (4-OH-APA) on arginine uptake (■) and arginase activity (●) in rabbit isolated AMΦ. Arginase activity was determined as described in Figure 1. Arginine uptake was studied by determining intracellular accumulation of radioactivity during 2 min incubation of AMΦ with [^{3}H]-L-arginine (37 kBq ml^{-1}, 0.1 μM). Test drugs, at the concentrations indicated, were present 30 min before and during incubation with [^{3}H]-L-arginine. Ordinate scale: arginase activity ([^{3}H]-L-ornithine accumulation) and arginine uptake (intracellular radioactivity) are expressed as % of the respective mean value observed in controls of the individual cell preparation. Results show mean of 5–7 experiments; vertical lines indicate s.e.mean.

on L-arginine turnover by iNOS was studied in rat AMΦ in which different levels of iNOS had been induced by culturing the cells in the absence or presence of 0.1 or 1 μg ml^{-1} LPS. As already shown previously (Hey *et al*., 1995), significant iNOS activity, as measured by the accumulation of [^{3}H]-L-citrulline, was already observed in rat AMΦ cultured in the absence of LPS (133 ± 11 d.p.m. $\times 1000$ per 2.5×10^6 AMΦ, Table 1).

Nevertheless, after culture in the presence of 0.1 or 1 μg ml^{-1} LPS an increase in [^{3}H]-L-citrulline accumulation of 37 and 99%, respectively, was observed (Table 1). In rat AMΦ cultured under the control conditions, the formation of [^{3}H]-L-ornithine (212 ± 8 d.p.m. $\times 1000$ per 2.5×10^6 AMΦ, Table 1) was significantly higher ($P < 0.01$) than that of [^{3}H]-L-citrulline. However, [^{3}H]-L-ornithine declined by 21 and 70%, when the AMΦ were cultured in the presence of 0.1 or 1 μg ml^{-1} LPS, respectively (Table 1).

In rat AMΦ, N$^\omega$-hydroxy-D,L-indospicine also inhibited the formation of [^{3}H]-L-ornithine and inhibitory effects of N$^\omega$-hydroxy-D,L-indospicine were observed in AMΦ cultured in the absence or presence of LPS (Table 1). Because of limited availability of material complete concentration-response curves were not obtained in these experiments. However, it appears that the potency of N$^\omega$-hydroxy-D,L-indospicine increased with the degree of induction of iNOS (Table 1). Thus, 10 μM N$^\omega$-hydroxy-D,L-indospicine reduced [^{3}H]-L-ornithine formation to 20%, 13% and 6.8% in AMΦ cultured in the absence or presence of 0.1 and 1 μg ml^{-1} LPS, respectively (Table 1). Morever, inhibition of arginase resulted in an increased formation of [^{3}H]-L-citrulline, i.e. a shift of the [^{3}H]-L-arginine metabolism to the NOS pathway. However, the degree of this shift was different under the different conditions. Thus, in control AMΦ only a relatively small part of the [^{3}H]-L-arginine not metabolized by the inhibited arginase appeared in the [^{3}H]-L-citrulline fraction, whereas in AMΦ cultured in the presence of LPS (0.1 and 1 μg ml^{-1} LPS) all the [^{3}H]-L-arginine not metabolized by the inhibited arginase was utilized by NOS, as indicated by the balanced increase in the [^{3}H]-L-citrulline fraction (Table 1). Thus, in AMΦ cultured in the presence of 0.1 μg ml^{-1} LPS the reduction of [^{3}H]-L-ornithine in the presence of 10 μM N$^\omega$-hydroxy-D,L-indospicine (Δ 146 d.p.m. $\times 1000$) was accompanied by an almost identical increase in [^{3}H]-L-citrulline (Δ 144 d.p.m. $\times 1000$). Likewise, in AMΦ cultured in the presence of 1 μg ml^{-1} LPS the reduction of [^{3}H]-L-ornithine in the presence of 10 μM N$^\omega$-hydroxy-D,L-indospicine (Δ 60 d.p.m. $\times 1000$) was accompanied by an increase in [^{3}H]-L-citrulline of the same magnitude (Δ 74 d.p.m. $\times 1000$). However, these figures show also that in absolute terms the shift from [^{3}H]-L-ornithine to [^{3}H]-L-citrulline caused by the arginase inhibitor was larger in AMΦ cultured in the presence of 0.1 μg ml^{-1} LPS (Δ about 145 d.p.m. $\times 1000$) than in AMΦ cultured in the presence of 1 μg ml^{-1} LPS (Δ about 60 – 70 d.p.m. $\times 1000$). This is explained by the fact that in AMΦ cultured in the presence of the higher concentration of LPS, the formation of [^{3}H]-L-ornithine was already substantially reduced in the absence of an exogenous arginase inhibitor (Table 1).

Discussion

N$^\omega$-hydroxy-D,L-indospicine and 4-hydroxy-D,L-phenylalanine have recently been described as potent and selective inhibitors of liver arginase (Custot *et al*., 1996; Vadon *et al*., 1996). MΦ, including AMΦ, are also known to express arginase activity (see Introduction). However, there is evidence for some heterogeneity of the arginase enzymes; the enzymes in non-liver tissues appear to be different from that in liver (Herzfeld & Raper, 1976; see Jenkinson *et al*., 1996). Nevertheless, the present results show that both drugs, N$^\omega$-hydroxy-D,L-indospicine and 4-hydroxyamidino-D,L-phenylalanine, are also potent inhibitors of the arginase pathway in AMΦ. They inhibited the formation of [^{3}H]-L-ornithine in rabbit isolated AMΦ with IC_{50} values of 2 and 45 μM, respectively. For comparison they inhibited rat liver arginase with IC_{50} values of 50 and 230 μM, respectively (Custot *et al*., 1996). Thus, they were apparently even more potent inhibitors of the arginase in AMΦ than of the liver enzyme. However, this apparent difference in potency might be caused by the different assay conditions. The potencies in the present study with intact cells may not be directly comparable with those obtained in an enzyme assay on liver cell homogenates. Nevertheless, in both test systems, N$^\omega$-hydroxy-D,L-indospicine was significantly more potent than 4-hydroxyamidino-D,L-phenylalanine.

In the present study arginase activity was measured in intact cells by determining the formation of [^{3}H]-L-ornithine during incubation with [^{3}H]-L-arginine. Thus, in the present experiments a reduction in [^{3}H]-ornithine formation could have been the result of an inhibition of [^{3}H]-L-arginine uptake, i.e. a reduced substrate supply. In AMΦ (Hey *et al*., 1996b), like in MΦ and many other cells (e.g. Sato *et al*., 1992; Bogle *et al*., 1992; 1996; see also White, 1985), L-arginine uptake is mediated via specific cationic amino acid transporters with the characteristics of the system y+. In the present experiments, 4-hydroxyamidino-D,L-phenylalanine caused a concentration-dependent inhibition of [^{3}H]-L-arginine uptake with a potency about 6 – 7 times lower than that to inhibit [^{3}H]-L-ornithine formation. Thus, an inhibitory effect on [^{3}H]-L-arginine uptake may have contributed to, but may not essentially account for the reduction in [^{3}H]-L-ornithine formation by 4-hydroxyamidino-D,L-phenylalanine. On the other hand, N$^\omega$-hydro-

Table 1 Effect of N$^\omega$-hydroxy-D,L-indospicine on the metabolism of [^{3}H]-L-arginine in rat AMΦ (cultured in the absence and presence of LPS) by NOS and arginase, as determined by the accumulation of [^{3}H]-L-citrulline and [^{3}H]-L-ornithine

	[^{3}H]-L-citrulline (d.p.m. $\times 1000$ per 2.5×10^6 cells)	*[^{3}H]-L-ornithine*	n
Controls	133 ± 11	212 ± 7.9	3
+ N$^\omega$-hydroxy-D,L-indospicine, 10 μM	186 ± 7.7*	43 ± 0.5**	3
+ N$^\omega$-hydroxy-D,L-indospicine, 30 μM	163 ± 1.4	16 ± 0.5**	3
LPS (0.1 μg ml^{-1})	$183 \pm 11^{+}$	168 ± 18	4
+ N$^\omega$-hydroxy-D,L-indospicine, 10 μM	327 ± 52*	22 ± 5.8*	5
+ N$^\omega$-hydroxy-D,L-indospicine, 30 μM	390 ± 53**	7.8 ± 1.3**	4
LPS (1 μg ml^{-1})	$265 \pm 16^{++}$	$64 \pm 4.3^{++}$	4
+ N$^\omega$-hydroxy-D,L-indospicine, 10 μM	339 ± 5.0*	4.4 ± 1.1**	3

AMΦ were obtained by bronchoalveolar lavage of rat isolated lungs and disseminated in culture dishes (2.5×10^6 cells per well). AMΦ were cultured for 20 h in the absence (control) or presence of 0.1 or 1 μg ml^{-1} LPS. Thereafter, AMΦ were incubated for 1 h in 1 ml Krebs-HEPES medium containing [^{3}H]-L-arginine (37 kBq, 0.1 μM). In the respective experiments, N$^\omega$-hydroxy-D,L-indospicine (at the concentrations indicated) was present 30 min before and during incubation with [^{3}H]-L-arginine. [^{3}H]-L-citrulline and [^{3}H]-L-ornithine accumulation in the incubation media was determined by h.p.l.c. followed by liquid scintillation spectrometry. Data are expressed as d.p.m. $\times 1000$ per 2.5×10^6 cells, means $\pm$ s.e.mean of *n* experiments. Statistical significance of differences: from the respective value in the absence of N$^\omega$-hydroxy-D,L-indospicine, *$P < 0.05$, **$P < 0.01$; from the controls, $^{+}P < 0.05$, $^{++}P < 0.01$.

xy-D,L-indospicine appears to be a specific inhibitor of arginase, lacking significant effects on the L-arginine transporter. N^{ω}-hydroxy-D,L-indospicine, up to a concentration which exceeded 50 fold the EC_{50} value for the inhibitory effect on [^{3}H]-L-ornithine formation, did not significantly affect [^{3}H]-L-arginine uptake. Therefore, N^{ω}-hydroxy-D,L-indospicine appeared to be more suitable than 4-hydroxyamidino-D,L-phenylalanine for experiments in which interactions between the arginase pathway and the NOS pathway were studied.

AMΦ can also express an inducible form of NOS which may compete with arginase for the common substrate, L-arginine. In previous studies it was already shown that induction of iNOS resulted in a reduced utilization of L-arginine by arginase (Hey *et al*., 1995; Hecker *et al*., 1995). The present experiments demonstrate that inhibition of arginase resulted in a shift of the [^{3}H]-L-arginine metabolism into the NOS pathway indicating that arginase, on the other side, can also limit utilization of L-arginine by iNOS. However, under the three different conditions studied (shown in Table 1), the impact of arginase on L-arginine utilization by iNOS appeared to be different, depending on the level of iNOS. (1) In AMΦ, cultured in the absence of LPS, the formation of [^{3}H]-L-ornithine was higher than that of [^{3}H]-L-citrulline, and after inhibition of arginase only about 20–30% of the [^{3}H]-L-arginine not metabolized by arginase was utilized by iNOS. Thus, under these conditions substrate competition between the NOS pathway and arginase pathway appears to be only of minor significance. (2) In AMΦ, cultured in the presence of 0.1 μg ml^{-1} LPS, the formation of [^{3}H]-L-citrulline was slightly increased in comparison to control AMΦ and this increase appeared to be at the expense of the [^{3}H]-L-ornithine fraction. Under these controls, after inhibition of arginase all the [^{3}H]-L-arginine not metabolized by this enzyme was utilized by iNOS, indicating that arginase was limiting the L-arginine supply for the NOS pathway, i.e. the NO synthesis. (3) In AMΦ, cultured in the presence of 1 μg ml^{-1} LPS, the formation of [^{3}H]-L-citrulline was further increased and this again was at the expense of the [^{3}H]-L-ornithine fraction, resulting in a marked reduction in [^{3}H]-L-ornithine. This apparent reduction in arginase activity may not be caused by a decline in arginase levels, as NOS inhibition completely restored [^{3}H]-L-ornithine formation to the control levels (Hey *et al*., 1995). After inhibition of arginase again, all the [^{3}H]-L-arginine not metabolized to [^{3}H]-L-ornithine appeared in the [^{3}H]-L-citrulline fraction. However, in absolute terms the shift of [^{3}H]-L-arginine to the NOS pathway was smaller than in the experiments in which the AMΦ had been cultured with 0.1 μg ml^{-1} LPS. These observations indicate that after marked induction of iNOS the limitation of NO synthesis by arginase became less prominent, because most of the L-arginine was already drawn toward the NOS pathway. Several mechanisms may account for the apparent prefered utilization of L-arginine by iNOS: (i) high levels of iNOS may produce significant amounts of the endogenous arginase inhibitor N^{ω}-hydroxy-L-arginine (Hecker *et al*., 1995); (ii) the affinity of L-arginine for NOS (K_m 3–30 μM, Stuehr *et al*., 1991; Hevel *et al*., 1991; Yui *et al*., 1991) is higher than that for arginase (K_m mmolar range, Kaysen & Strecker, 1973; Meijer *et al*., 1990).

In conclusion, N^{ω}-hydroxy-D,L-indospicine is a potent and specific inhibitor of arginase in AMΦ. In cells in which, in addition to arginase, iNOS is expressed, competition between both enzymes for the common substrate L-arginine may be of significance and inhibition of arginase can enhance L-arginine utilization by the NOS pathway, i.e. increase NO synthesis. The extent of this shift in the L-arginine metabolism after arginase inhibition appears to depend on the level of iNOS expression.

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