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RESEARCH COMMUNICATION

L-Arginine transport is increased in macrophages generating nitric oxide

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Transport of L-arginine and nitrite production were examined in the murine macrophage cell line J774. Bacterial lipopolysaccharide (LPS) induced a dose- and time-dependent stimulation of nitrite production, which was further increased in the presence of interferon-γ. Nitrite synthesis was absolutely dependent on extracellular L-arginine and inhibited in the presence of L-lysine or L-ornithine. In unactivated J774 cells L-arginine transport was saturable, with an apparent K_m of 0.14 ± 0.04 mM and $V_{max.}$ of 15 ± 2 nmol/h per 10^6 cells. LPS (1 μg/ml) induced a time-dependent stimulation of L-arginine transport, and after 24 h the $V_{max.}$ increased to 34 ± 2 nmol/h per 10^6 cells. These findings indicate that activation of J774 cells with LPS produces an increase in both L-arginine transport and nitrite synthesis. The elevated rate of L-arginine transport in activated J774 cells may provide a mechanism for sustained substrate supply during enhanced utilization of L-arginine for the generation of NO.

INTRODUCTION

Macrophages play an important role in the regulation of acquired and innate immunity [1–3], in part by the secretion of cytokines including interleukin-1, tumour necrosis factor and interferon-γ. Activated macrophages also synthesize nitric oxide (NO), and it is this radical which is responsible for many of their cytotoxic effects [4–6]. Synthesis of NO in these cells is mediated by a Ca^{2+}-independent NO synthase, which is induced after exposure to cytokines and/or bacterial lipopolysaccharide (LPS) [7,8]. Recent studies have demonstrated a requirement for extracellular L-arginine for the production of NO from activated macrophages [9–12], and L-arginine can be critical for macrophage function during infection or at local sites of tissue injury [13,14].

In this study we have investigated the induction of L-arginine transport and nitrite production by LPS and interferon-γ in the macrophage cell line J744 to test the hypothesis that L-arginine transport is regulated in parallel with production of NO.

METHODS

Cell culture

The murine monocyte/macrophage cell line J774 was obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C., Porton Down, Wiltshire, U.K.). J774 cells were maintained in continuous culture in T75 tissue-culture flasks in Dulbecco's modified Eagle's medium containing 0.4 mM-L-arginine and supplemented with 4 mM-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% (v/v) foetal-calf serum. Cells were harvested by gentle scraping and passaged every 3–6 days by dilution of a suspension of the cells 1:10 in fresh medium.

Measurement of L-arginine transport

J774 cells were plated at a density of 10^5 cells per well in 96-well microtitre plates and allowed to adhere for 2 h. Thereafter medium was replaced either with fresh medium or with medium containing LPS and/or other compounds for specific time periods (1–24 h). After incubation, cells were rinsed twice with a modified Hepes-buffered Krebs solution of the following composition (mM): NaCl, 131; KCl, 5.5; $MgCl_2$, 1; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1; D-glucose, 5.5; Hepes, 20; pH 7.4, 37 °C. L-[^{3}H]Arginine was added to the monolayers, and transport was measured over the next 5 min. Incubations were terminated by placing the plates on melting ice and rinsing cells with 3×200 μl of ice-cold Dulbecco's phosphate-buffered saline (138 mM-NaCl/2.6 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4) containing 10 mM unlabelled L-arginine. In some experiments an extracellular reference tracer, D-[^{14}C]mannitol, was included in the incubation medium. The efficiency of the washing procedure was confirmed by a recovery of D-[^{14}C]mannitol < 0.01% of that applied.

Cell protein was determined with the Bio-Rad reagent, and cell number was estimated by using a haemocytometer. Radioactivity in formic acid digests of the cells was determined by liquid-scintillation counting. Uptake was then calculated and expressed in units of nmol/h per 10^6 cells.

Measurement of nitrite formation

Production of NO by NO synthase was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction [6,15]. J774 cells were seeded into 96-well microtitre plates and exposed to LPS and/or other compounds as described above. After a specified incubation time, a portion (100 μl) of the medium was removed, mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthyl-ethylenediamine dihydrochloride/2.5% H_3PO_4) and then incubated at room temperature for 10 min. The A_{560} was read with a Multiskan II plate reader (Titertek). Nitrite concentration in the medium was determined with $NaNO_2$ as a standard.

Isolated NO synthase

J774 cells in suspension culture were activated with LPS

Abbreviations used: LPS, lipopolysaccharide.

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Table 1. Concentration-dependent stimulation of nitrite production and increase in L-arginine transport in J774 cells

Cells were exposed to LPS (0.1–10 μg/ml) or LPS and interferon-γ (100 units/ml). Accumulated nitrite production was measured 24 h later as described in the Methods section. Uptake of L-[^{3}H]arginine (0.25 mM) was determined over 5 min in cells exposed to either LPS or LPS and interferon-γ for the previous 24 h. Results are means ± S.E.M. of 18 replicate measurements from three experiments: *$P < 0.05$ versus control values, †$P < 0.05$ versus values obtained in the presence of LPS alone (paired t test).

	Nitrite release (nmol/24 h per 10^6 cells)	L-Arginine uptake (nmol/h per 10^6 cells)
Control		
LPS	0.73 ± 0.6	26.5 ± 0.9
0.1	16.2 ± 2.9*	41.1 ± 2.6*
1.0	27.1 ± 3.5*	40.2 ± 2.9*
10.0	37.3 ± 5.5*	47.8 ± 2.6*
Interferon-γ (100 units/ml) + LPS		
0.1	49.3 ± 6.6†	40.0 ± 3.0
1.0	56.0 ± 7.2†	35.8 ± 2.9
10.0	70.5 ± 5.3†	38.0 ± 3.6†

(1 μg/ml) and interferon-γ (100 units/ml) for 24 h. Subsequent measurement of Ca^{2+}-independent inducible NO synthase activity was performed by a direct spectrophotometric assay as described previously [7].

Materials

All reagents for cell culture except foetal-calf serum (Globepharm, Esher, Surrey, U.K.) were from Gibco. LPS extracted from *Escherichia coli* (055:B5) was obtained from Difco, Detroit, MI, U.S.A. Recombinant murine interferon-γ was from Holland Biotechnologies. Other chemicals were from Sigma or BDH and of the highest grade obtainable. Radioactive tracers L-[2,3-^{3}H]arginine (53 Ci/mmol) and D-[1-^{14}C]mannitol (50 mCi/mmol) were obtained from New England Nuclear.

Statistics

All values are means ± S.E.M. of n experiments. Statistical analysis was performed with a Student's t test, with $P < 0.05$ considered statistically significant.

RESULTS

Induction of nitrite formation and L-arginine transport by LPS and interferon-γ

Nitrite release from unstimulated J774 cells incubated for 24 h in normal culture medium (containing 0.4 mM-L-arginine) was below the limit of detection (< 0.01 nmol). Inclusion of LPS in the incubation medium resulted in a dose-dependent stimulation of nitrite formation, which was further increased by interferon-γ (100 units/ml; Table 1). Nitrite production was time-dependent, becoming detectable after 8 h (11 ± 1 nmol/10^6 cells) and increasing to 75 ± 6 nmol/10^6 cells after 24 h exposure to LPS and interferon-γ ($n = 3$; Fig. 1*a*).

Transport of L-arginine in control or LPS-treated (1 μg/ml; 24 h) J774 cells was linear ($r = 0.99$) for up to 10 min ($n = 3$) and temperature-dependent (results not shown). Hence, subsequent measurements were carried out over a 5 min incubation period. Table 1 shows that, although L-arginine transport was elevated by LPS, this was not concentration-dependent over the range 0.1–10 μg/ml, and addition of interferon-γ (100 units/ml) did not further enhance the effects of LPS. Transport was significantly elevated after 4 h of activation, and this increase was maintained above control levels over a 24 h period (Fig. 1*b*).

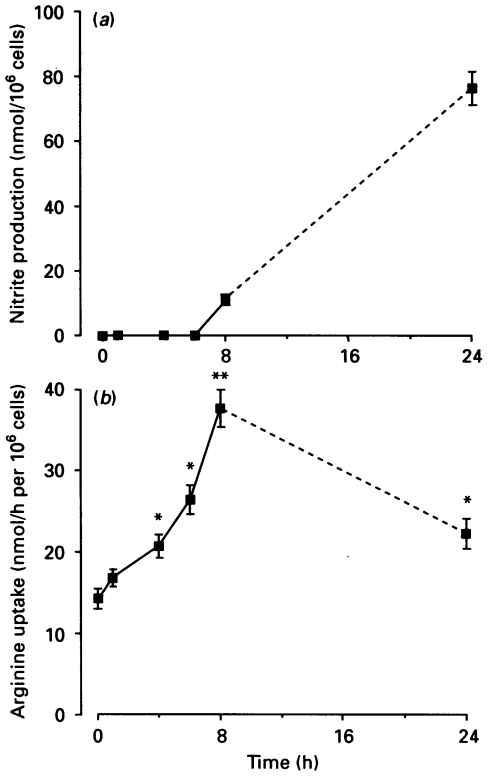

Fig. 1. Time course of induction of L-arginine transport and nitrite synthesis

(*a*) Cells were treated with LPS (1 μg/ml) and interferon-γ (100 units/ml) for 0–24 h. Accumulated nitrite production was measured. (*b*) Transport of L-[^{3}H]arginine (0.25 mM) was measured during a 5 min incubation period after activation with LPS (1 μg/ml) and interferon-γ (100 units/ml). Results are means ± S.E.M. of 3 experiments: *$P < 0.05$, **$P < 0.01$ versus control values (paired t test).

Role of extracellular L-arginine in nitrite synthesis

The dependency of nitrite formation on extracellular L-arginine was studied by incubating J774 cells for 24 h with LPS (1 μg/ml) and interferon-γ (100 units/ml) in the presence of increasing concentrations of L-arginine (0–3 mM; Fig. 2). In the absence of L-arginine, activated J774 cells did not synthesize nitrite. However, L-arginine caused a concentration-dependent increase in nitrite production, which reached a maximum at concentrations above 0.3 mM. The $K_{0.5}$ for nitrite production was 37 ± 4 μM, with a $V_{max.}$ of 81 ± 2 nmol/24 h per 10^6 cells ($n = 3$).

L-Lysine or L-ornithine (0.5–10 mM) inhibited the transport of

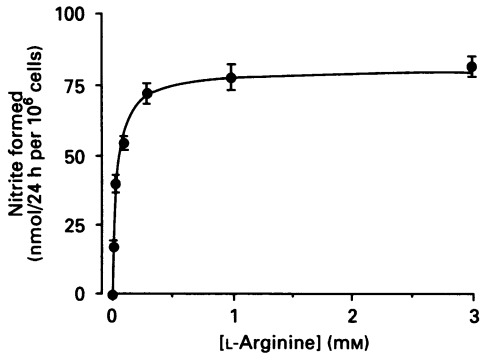

Fig. 2. Dependency of nitrite formation on extracellular L-arginine

Cells were treated with LPS (1 μg/ml) and interferon-γ (100 units/ml) for 24 h in the presence of increasing concentrations of L-arginine (0–3 mM) and nitrite production was determined. Results are means ± S.E.M. of 3 experiments.

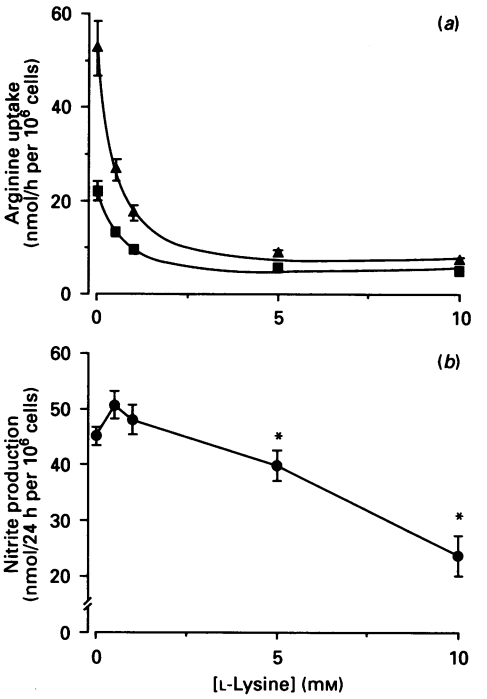

Fig. 3. Inhibition of L-arginine transport and nitrite production by L-lysine

The effect of L-lysine (0–10 mM) on the transport of L-arginine (0.1 mM) was assessed in control cells (■) or cells activated with LPS (1 μg/ml) and interferon-γ (100 units/ml; ▲). (*b*) J774 cells were activated with LPS/interferon-γ in the presence of L-arginine (0.1 mM) and increasing concentrations of L-lysine (0–10 mM); nitrite production was determined 24 h later. Results are means ± S.E.M. of 3 experiments: $*P < 0.05$ (paired *t* test).

L-arginine (0.1 mM; Fig. 3*a*). Calculated K_i values for inhibition of L-arginine transport (0.1 mM) were 0.33 ± 0.03 and 0.36 ± 0.02 mM for L-lysine and L-ornithine respectively. In addition, these compounds caused a concentration-dependent inhibition of nitrite production by activated J774 cells. At 10 mM, L-lysine (Fig. 3*b*) or L-ornithine inhibited nitrite production by 48 ± 2% or 37 ± 1% respectively ($n = 3$).

Further experiments were performed to assess whether L-lysine and L-ornithine were inhibitors of the isolated NO synthase in J774 cells. In the presence of L-arginine (0.03 mM), L-lysine (10 mM) or L-ornithine (10 mM) inhibited NO synthesis by 26 ± 3% and 19 ± 3% respectively ($n = 3$).

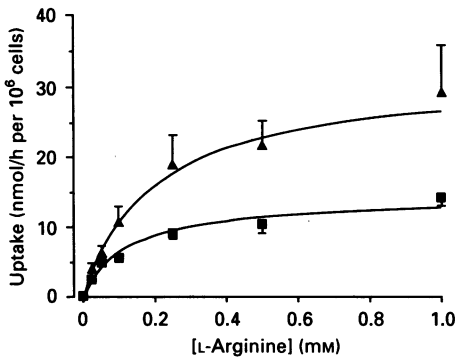

Fig. 4. Kinetics of L-arginine transport in control and LPS-treated J774 cells

L-Arginine transport was examined in control cells (■) and after activation with LPS (1 μg/ml) for 24 h (▲). Results are means ± S.E.M. of 4 experiments in which rectangular hyperbolae were fitted to the mean influx data weighted for the reciprocal of the S.E.M. at each concentration.

Kinetics of L-arginine transport in LPS-treated J774 cells

The kinetics of L-arginine transport were examined over the concentration range 0.025–1 mM. Transport of L-arginine by unstimulated J774 cells was saturable, with an apparent K_m of 0.14 ± 0.04 mM and $V_{max.}$ of 15 ± 2 nmol/h per 10^6 cells ($n = 4$; Fig. 4). Activation of cells with LPS (1 μg/ml) for 24 h increased the $V_{max.}$ to 34 ± 2 nmol/h per 10^6 cells. Treatment of J774 cells with a combination of LPS (1 μg/ml) and interferon-γ (100 units/ml) did not further increase the $V_{max.}$ of transport (results not shown).

DISCUSSION

Our results demonstrate that activation of macrophages results in a substantial enhancement of L-arginine transport. Activation of J774 cells with LPS was followed by accumulation of nitrite in the culture medium, which was dependent on the presence of extracellular L-arginine. The $K_{0.5}$ for this process was 37 μM, a value similar to that previously reported in freshly isolated murine macrophages [11] and similar to values obtained with NO synthase prepared from the cytosol of activated macrophages [16,17]. These observations confirm reports in other activated macrophage cells or cell lines [7,12,18], and demonstrate that arginine availability is rate-limiting for the generation of NO and hence nitrite. These findings suggest that the transport of L-arginine may be an important regulatory step in the pathway for NO generation. Stimulation of J774 cells with a combination of LPS and interferon-γ resulted in a greater synthesis of nitrite than with LPS alone, but did not further stimulate uptake. In addition the dose-dependency was different, suggesting that the induction of NO synthase enzyme and L-arginine transporter may be regulated differentially. There is evidence that inactivation of cellular processes occurs during elevated NO production [14], and this may account in part for the decline of L-arginine transport after 8 h of LPS/interferon-γ treatment.

Little is known about the characteristics or mechanisms regulating L-arginine transport in macrophages. Rabbit lung macrophages transport L-lysine via a carrier-mediated system [19–21], and recent studies in mouse peritoneal macrophages have reported that L-lysine is transported via the system y^+ amino acid transporter [22] and uptake is stimulated after exposure to LPS [23]. In our study, entry of L-arginine into resting and activated J774 cells was mediated by a saturable system. Kinetic analysis revealed that over the concentration

range 0.025–1 mM-L-arginine transport was mediated via a single transport system. This transporter was sensitive to inhibition by L-lysine and L-ornithine. It is therefore likely that uptake of L-arginine in J774 cells is mediated by system y^+, and more work is needed to substantiate the presence of this transporter in J774 cells. The increase in $V_{max.}$ without significant change in the K_m also suggests that stimulation of arginine uptake by LPS was due to increased synthesis and expression of the y^+ carrier protein.

In other studies, LPS-stimulated myeloid leukaemia cells showed no evidence for an increase in the transport of cationic amino acids [24]. In contrast, immunological activation of peritoneal macrophages enhanced leucine transport [25], and a decreased rate of lysine uptake was reported to occur during a 2 h period after peritoneal-macrophage collection [26] or during oxidant exposure [27]. In some cells L-arginine transport is mediated via the γ-glutamyl cycle [28], whose activity can be modulated after macrophage activation [29,30]. These results suggest that multiple mechanisms are responsible for the regulation of amino acid transport in macrophages. The specificity of the effects of LPS on the induction of cationic amino acid transport in J774 cells remains to be determined.

L-Lysine and L-ornithine decreased, in a concentration-dependent manner, both the production of nitrite by activated J774 cells and the transport of L-arginine. The L-arginine transporter was apparently more sensitive to inhibition than was the production of nitrite, though it should be pointed out that transport was calculated from rapid uptake studies over 5 min, whereas nitrite production was monitored over 24 h. Thus, during the 24 h incubation period in the presence of L-lysine or L-ornithine, it is likely that these inhibitors of L-arginine transport are metabolized by the macrophages, leading to a decrease in the concentration of inhibitor to which the cells are exposed to during the incubation period. Also, although L-lysine and L-ornithine inhibit L-arginine transport substantially, there is still measurable transport of L-arginine ($\sim$ 5 nmol/h per 10^6 cells), which over a 24 h period may be utilized for the production of NO. In addition to these factors, we also found that both L-lysine and L-ornithine at high concentrations ($>$ 5 mM) have a direct inhibitory effect on NO synthase activity. These observations complicate the interpretation of the effects of these inhibitors, and more definitive studies are needed to determine the effects on NO release over shorter time periods.

At sites of macrophage infiltration *in vivo* there is a rapid depletion of L-arginine from the surrounding extracellular fluid [31,32]. This can result from metabolic conversion of arginine either into NO and citrulline, or metabolism to ornithine by arginase [11]. A recent study investigating L-arginine metabolism in activated murine macrophages indicated that approx. 70% of cellular L-arginine metabolism occurs via arginase and the remaining 30% via NO synthase [11]. In our experiments nitrite synthesis occurred at a rate of 3.2 nmol/h per 10^6 cells (see Fig. 2*a*) after induction with LPS and interferon-γ, indicating that total cellular arginine utilization may be at least 10 nmol/h per 10^6 cells. At plasma concentrations of L-arginine (approx. 0.1–0.2 mM) activated macrophages would transport 11–15 nmol/h per 10^6 cells (see Fig. 4), a value similar to the rate of utilization of L-arginine. Thus arginine transport is likely to be a rate-limiting step for the generation of NO by activated macrophages. Selective inhibition of arginine uptake may therefore provide a mechanism to limit the cytotoxicity of activated macrophages.

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