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Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells

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1 Effects of dexamethasone on induction of nitric oxide (NO) synathase and L-arginine transport by lipopolysaccharide (LPS) were examined in a murine cultured macrophage cell line J774. Metabolism of L-arginine to L-citrulline and subsequent changes in intracellular amino acids pools were correlated with changes in nitrite production.

2 Despite a high intracellular concentration of arginine in activated J774 cells, LPS (1 $\mu g\,ml^{-1}$, 8 h) induced a 2.4 fold increase in arginine transport. Treatment of cells with cycloheximide (1 $\mu g\,ml^{-1}$) inhibited the time-dependent (1–8 h) induction of NO synthase and arginine transport mediated by LPS.

3 Induction of NO synthase by LPS (1 $\mu g\,ml^{-1}$, 24 h) alone was accompanied by a marked increase in arginine utilisation leading to decreased intracellular arginine levels and elevated intracellular and extracellular L-citrulline levels. These changes were further enhanced in the presence of interferon-γ (IFN-γ, 100 units ml^{-1}, 24 h).

4 Dexamethasone (1 μM) abolished the increases in both nitrite and citrulline production induced by LPS alone but only partially reversed the combined effects of LPS and IFN-γ. In contrast, treatment of cells with dexamethasone (10 μM) had no effect on the LPS-mediated induction of arginine transport or the decrease in intracellular arginine concentration.

5 We conclude that induction of arginine transporter activity in LPS-stimulated J774 cells involves *de novo* synthesis of carrier proteins, which increases transport of exogenous arginine during enhanced NO production. Moreover, the intracellular signalling pathways mediating induction of arginine transport and of NO synthase by LPS in activated macrophages diverge, since only the latter is sensitive to dexamethasone.

Keywords: Macrophage; nitric oxide; L-arginine transport; bacterial lipopolysaccharide; dexamethasone; interferon-γ; cycloheximide; intracellular amino acid concentrations; L-arginine; L-citrulline; L-ornithine

Introduction

Metabolism of arginine to nitric oxide (NO) in macrophages is mediated by a Ca^{2+}/calmodulin-insensitive NO synthase enzyme, which is induced following activation of these cells with bacterial endotoxin and/or cytokines (Hibbs *et al.*, 1987; 1988; Iyengar *et al.*, 1987; Stuehr & Marletta, 1987; Marletta *et al.*, 1988). Expression of this enzyme is time-dependent, known to involve *de novo* protein synthesis and can be inhibited by glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990; Assreuy & Moncada, 1992). The sustained production of NO is critically dependent on the presence of extracellular arginine (Drapier & Hibbs, 1988; Granger *et al.*, 1990; Takema *et al.*, 1991; Bogle *et al.*, 1992a; Assreuy & Moncada, 1992).

We and another group have recently shown that activation of either a murine macrophage cell line J774 or murine peritoneal macrophages with LPS results in a marked increase in arginine uptake (Bogle *et al.*, 1992a; Sato *et al.*, 1992). In both studies uptake was mediated by a carrier resembling the cationic amino acid transporter system y^+ (White, 1985). We found that the V_{max} for arginine uptake doubled when J774 cells were activated, with no significant alteration in K_t (0.14 mM), suggesting that enhanced uptake of arginine reflects increased expression of carrier protein (Bogle *et al.*, 1992a). Sato *et al.* (1992), did not perform comparative kinetic studies, but stated that increased arginine uptake could be blocked by treatment with cycloheximide. We also demonstrated that inhibition of arginine uptake in activated J774 cells by ornithine or lysine (alternative system y^+ substrates) resulted in a concentration-dependent inhibition of NO production (Bogle *et al.*, 1992a). Activation of macrophages by LPS seems to involve induction of the system y^+ transporter, and enhanced arginine transport appears to be essential for these cells to synthesize NO, despite several reports that activated macrophages apparently contain adequate intracellular levels of arginine (Hecker *et al.*, 1990; Bogle *et al.*, 1992b; Assreuy & Moncada, 1992).

In the present study we have therefore determined whether enhanced arginine transport in J774 cells is dependent on new protein synthesis and investigated whether similar signal transduction pathways regulate induction of arginine transport and NO synthase. We have also examined in more detail alterations in intracellular amino acid pool sizes in activated J774 cells, to gain further information about the relative roles of intracellular and extracellular arginine in NO production.

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., Wiltshire). J774 cells were maintained in continuous culture in T75 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 0.4 mM L-arginine and supplemented with 4 mM glutamine, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$) and 10% 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.

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Measurement of L-arginine transport

J774 cells were plated at a density of 10^5 cells per well in 96-well microtiter plates and allowed to adhere for 2 h. Thereafter medium was replaced with either fresh DMEM or DMEM containing lipopolysaccharide (LPS, 1 μg ml^{-1}) alone or with LPS plus either cycloheximide (1 μM) or dexamethasone (10 μM) for specified time periods (1–8 h). After each 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 (1 μCi ml^{-1}, 0.25 mM) was added to the monolayers and transport measured over the next 5 min, since in our previous study uptake of L-arginine was shown to be linear for 10 min (Bogle *et al.*, 1992a). Incubations were terminated by placing the plates on melting ice and rinsing cells three times with 200 μl ice-cold Dulbecco's phosphate buffered saline (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 in the cell lysate of $<0.01\%$ of D-[^{14}C]-mannitol applied. Cell protein was determined with the BioRad reagent and cell number was estimated in a haemocytometer. Radioactivity in formic acid digests of the cells was determined by liquid scintillation counting. Uptake was expressed in units of nmol/10^6 cells h^{-1}.

Measurement of nitrite production as an assay of NO release

NO production by J774 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction (Green *et al.*, 1982). J774 cells were seeded into 96-well microtiter plates (10^5 cells/well) and exposed to LPS (1 μg ml^{-1}) or LPS and IFN-γ (100 units ml^{-1}) for 1–24 h. In some experiments cycloheximide (1 μM) or dexamethasone (1 μM) was added to the culture medium. After a specified incubation time, an aliquot (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. Absorbance was read at 560 nm using a Multiskan II plate reader (Titertek). Nitrite concentration in the medium was determined with sodium nitrite used as a standard.

Analysis of intracellular amino acid concentrations in J774 cells

J774 cells were seeded into 24 well plates (10^6 cells/well) and allowed to adhere for 2 h. Thereafter the medium was replaced with either fresh medium or medium containing LPS and/or other compounds. Following a 24 h incubation period, the medium was collected, centrifuged at 10,000 *g* for 5 min and stored at − 70°C for subsequent analysis by high performance liquid chromatography (h.p.l.c.). Cells were rinsed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), lysed with 0.5 ml methanol and cell lysates stored at − 70°C for h.p.l.c. analysis. The following were added to each standard or deproteinised sample (20 μl): 80 μl borate buffer (0.2 M, pH 9), 20 μl internal standard (0.1 mM homoserine in distilled water), 20 μl iodoacetic acid reagent (0.74 g iodoacetic acid, 0.62 g boric acid in 100 ml water; pH 9.5), and 20 μl *o*-phthaldialdehyde reagent (80 mg dissolved in 2 ml ethanol and 0.2 ml 2-mercaptoethanol in 100 ml distilled water). The tube was vortex-mixed and 1 min later a 50 μl aliquot of this mixture was injected into a Hypersil Ultratechsphere ODS-5 μm h.p.l.c. column (Jones Chromatography, Mid-Glamorgan) attached to a Beckman 112 h.p.l.c. system (Beckman RIIC Ltd., Bucks.).

Amino acids were separated by a two-solvent gradient system delivered to the column at a flow rate of 2 ml min^{-1} (Baydoun *et al.*, 1990). Upon elution amino acid derivatives were detected by use of a Gilson Spectra-Glo Fluorescence detector (Gilson Medical Laboratories Inc., Wisconsin, U.S.A.) at an excitation wavelength of 388 nm and emission cut off filter of 425 nm. Amino acid concentrations were calculated from peak areas with reference to the internal standard homoserine and the area of an amino acid in a standard mixture containing 0.1 mM concentrations of each amino acid. Peak areas were integrated using a Shimadzu CR1B integrator (Shimadzu Corporation, Tokyo, Japan). Intracellular amino acid concentrations are expressed in mmol l^{-1}, where the volume is the intracellular water space (1 ± 0.1 pl, $n = 4$) measured as described by Kletzien *et al.* (1975).

Measurement of L-leucine incorporation into protein

Protein synthesis was estimated by monitoring incorporation of L-[^{3}H]-leucine into J774 cell protein. Cells monolayers were incubated with L-[^{3}H]-leucine (5 μCi ml^{-1}) for 8 h, rinsed twice with phosphate buffered saline, and then 200 μl trichloroacetic acid (5%) were added to precipitate cellular protein. Pellets were washed with methanol and formic acid digests were transferred to scintillation vials for liquid scintillation counting.

Materials

All reagents for cell culture, except foetal calf serum (Globe Laboratories Ltd), were from Gibco. LPS extracted from

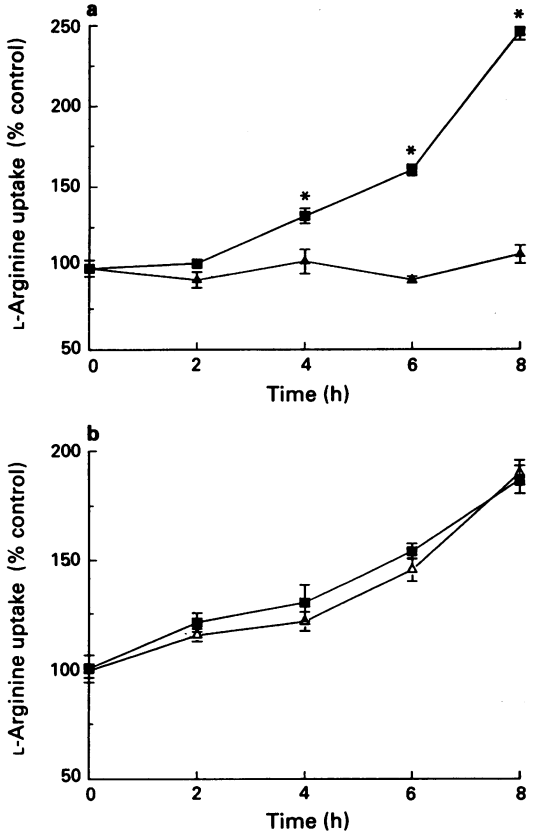

Figure 1 Effects of cycloheximide or dexamethasone on arginine uptake in J774 cells activated with lipopolysaccharide (LPS). Cells were stimulated with LPS (1 μg ml^{-1}) for increasing time periods (0–8 h) (a) in the absence (■) or presence of cycloheximide (▲, 1 μM) and (b) in the absence (■) or presence of dexamethasone (△, 10 μM). Uptake of L-[^{3}H]-arginine (0.25 mM) was then measured over a 5 min interval during incubation of cells with a HEPES-buffered amino acid-free Krebs solution (see Methods). Values denote the mean ± s.e. of replicate measurements in 3 separate experiments. *$P < 0.05$, Student's unpaired *t* test.

Escherichia coli (serotype 055:B5) was obtained from Difco, Michigan, U.S.A. IFN-γ (specific activity 100,000 units ml^{-1}; Holland Biotechnologies) was a gift from Wellcome Research Laboratories, Beckenham. Dexamethasone (Sigma, U.K.) was dissolved in 100% ethanol and diluted for use in culture medium at a final ethanol concentration of 0.01%. Other chemicals were from Sigma or BDH and of the highest grade obtainable. The radioactive tracers L-[2,3-^{3}H]-arginine (53 Ci $mmol^{-1}$), D-[1-^{14}C]-mannitol (50 mCi $mmol^{-1}$) and L-[3,4,5-^{3}H]-leucine (147 Ci $mmol^{-1}$) were obtained from NEN, Dreieich, Germany.

Statistics

All values are means ± s.e. of n experiments. Statistical analysis was performed using Student's t test with $P<0.05$ considered statistically significant.

Results

Effects of cycloheximide or dexamethasone on arginine transport and NO synthase in LPS-activated J774 cells

J774 cells were incubated with LPS (1 μg ml^{-1}) in the presence or absence of either cycloheximide (1 μM) or dexamethasone (10 μM) for up to 8 h and transport of arginine was then measured over a 5 min interval. Incubation of cells with LPS for 8 h increased transport from a basal value of 10.3 ± 1.8 to 24.3 ± 2.1 nmol per 10^6 cell h^{-1} ($n = 3$) and nitrite production to a maximum of 11.2 ± 1.4 nmol per 10^6 cells 8 h^{-1} ($n = 3$). Treatment with cycloheximide (1 μM, 8 h), a concentration which reduced the incorporation of L-[^{3}H]-leucine into cell protein by 85 ± 2% ($n = 10$), prevented induction of NO synthase, and abolished the time-dependent increase in arginine uptake without reducing uptake below control levels (Figure 1a). Exposure of J774 cells to cycloheximide for longer than 8 h caused cell loss from the culture wells.

As shown in Figure 1b, induction of arginine transport by LPS (1 μg ml^{-1}) was unaffected by co-incubation of J774 cells with a maximum concentration of dexamethasone (10 μM). In contrast, dexamethasone treatment resulted in substantial inhibition of NO synthase, reducing nitrite production from 11.2 ± 1.4 to 2.0 ± 0.3 nmol per 10^6 cells 8 h^{-1} ($n = 3$), confirming previous findings in cultured J774 cells (Di Rosa *et al.*, 1990; Assreuy & Moncada, 1992).

Amino acid concentrations in activated J774 cells

Chromatographic analyses of cell lysates obtained from untreated J774 cells revealed a high concentration of arginine with only trace amounts of citrulline (Table 1). Following activation of cells with LPS (1 μg ml^{-1}, 24 h), intracellular citrulline levels increased 1.5 fold. It should be noted that this increase dramatically underestimates the total production of citrulline, since there was a 5 fold accumulation of this amino acid in the extracellular medium (Table 2). Stimulation of cells with LPS (1 μg ml^{-1}) and IFN-γ (100 units ml^{-1}) resulted in greater increases in intracellular and extracellular concentrations of citrulline. Treatment with IFN-γ alone had no significant effect on intracellular citrulline (Table 1) and did not induce nitrite production (data not shown).

Although dexamethasone (1 μM, 24 h) completely inhibited the elevation of intracellular citrulline induced by LPS, it inhibited by only one third the production of nitrite and citrulline induced by a combination of LPS and IFN-γ (Figure 2). Increases in citrulline and nitrite accumulation following activation (Figure 2) were accompanied by parallel decreases in intracellular arginine levels (Table 1). LPS (1 μg ml^{-1}) alone reduced cellular arginine concentrations by 26%, whilst addition of IFN-γ (100 units ml^{-1}) and LPS reduced the intracellular pool by 57%, and also decreased medium concentrations of arginine by 56% (Table 2). These

Table 1 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on intracellular arginine, citrulline and ornithine concentrations in J774 cells

Condition	*Intracellular concentration* (mM) *L-Arginine*	*L-Citrulline*	*L-Ornithine*
Control	3.50 ± 0.30	0.11 ± 0.01	0.25 ± 0.05
LPS (1 μg ml^{-1})	2.61 ± 0.06*	0.17 ± 0.02*	0.31 ± 0.05
IFN-γ (100 units ml^{-1})	2.42 ± 0.36	0.08 ± 0.01	0.18 ± 0.01
LPS + IFN-γ (100 units ml^{-1})	1.50 ± 0.18*	0.35 ± 0.03*	0.58 ± 0.16
LPS + dexamethasone (1 μM)	2.69 ± 0.16*	0.10 ± 0.02	0.65 ± 0.18
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	1.77 ± 0.18*	0.23 ± 0.02*	0.51 ± 0.12

J774 cells were cultured to confluence in 24-well plates (10^6 cells/well) and treated for 24 h with combinations of LPS, IFN-γ and dexamethasone. Amino acids in lysates from control and treated cells were measured by reversed phase h.p.l.c. and the free amino acid concentrations expressed relative to an intracellular water space of 1 pl/cell. Values denote the mean ± s.e. of 4 separate experiments. *$P<0.05$, Student's unpaired t test.

Table 2 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on arginine, citrulline and ornithine concentrations in culture medium

Condition	*Culture medium concentration* (mM) *L-Arginine*	*L-Citrulline*	*L-Ornithine*
Control	0.40 ± 0.10	0.02 ± 0.005	0.05 ± 0.01
LPS (1 μg ml^{-1})	0.38 ± 0.06	0.10 ± 0.01*	0.08 ± 0.02
IFN-γ (100 units ml^{-1})	0.43 ± 0.05	0.06 ± 0.005	0.07 ± 0.01
LPS + IFN-γ (100 units ml^{-1})	0.17 ± 0.01*	0.32 ± 0.03*	0.06 ± 0.01
LPS + dexamethasone (1 μM)	0.33 ± 0.04	0.04 ± 0.005	0.05 ± 0.01
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	0.23 ± 0.04*	0.21 ± 0.01*	0.08 ± 0.02

Details as in legend to Table 1. Culture medium (DMEM containing 10% foetal calf serum) from control and treated cells were deproteinised and free amino acid concentrations were determined by reversed phase h.p.l.c. Values are the mean ± s.e. of measurements in 4 experiments. *$P<0.05$, Student's unpaired t test.

changes in arginine concentration were unaffected by dexamethasone, despite inhibition of nitrite and citrulline production, demonstrating that arginine is utilised by other pathways activated by LPS/IFN-γ. The fact that intracellular ornithine levels tended to increase in the presence of LPS/IFN-γ suggests increased metabolism via arginase. Furthermore, dexamethasone had no significant effect on the production of ornithine in LPS/IFN-γ activated cells (Table 1).

It is worth noting that treatment of J774 cells with either LPS (1 μg ml^{-1}) or IFN-γ (100 units ml^{-1}) alone for 24 h reduced the total intracellular amino acid pool by 33% or 44%, respectively (Table 3). These changes in intracellular amino acid concentrations were enhanced further when cells were incubated with LPS and IFN-γ for 24 h (Table 3). Dexamethasone had no significant effects on LPS/IFN-γ induced changes in the intracellular amino acid pool or medium concentrations.

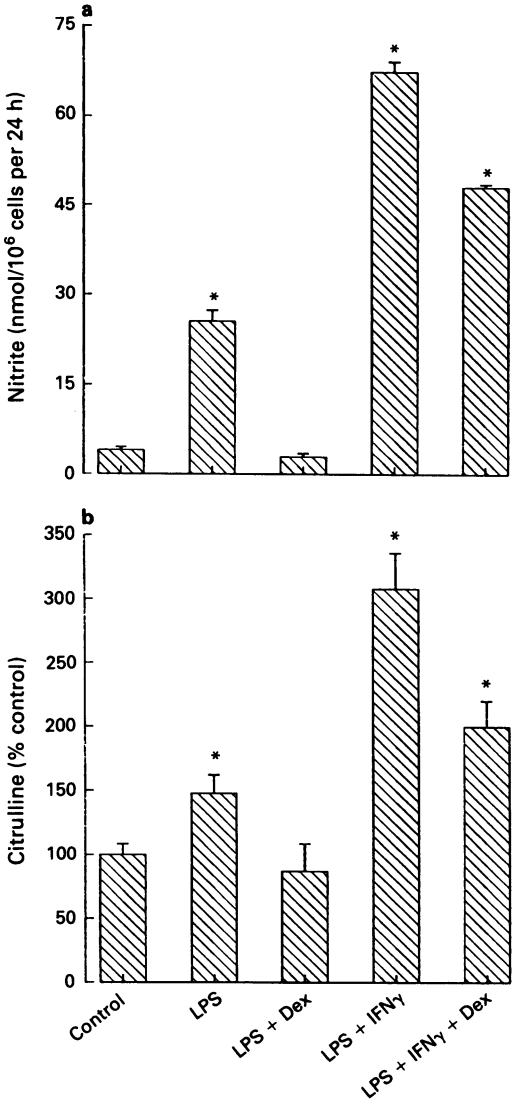

Figure 2 Effects of dexamethasone on nitrite and citrulline production in J774 cells activated with lipopolysaccharide (LPS) or LPS plus interferon-γ (IFN-γ). Cells were incubated with combinations of LPS (1 μg ml^{-1}), IFN-γ (100 units ml^{-1}) and dexamethasone (1 μM) for 24 h. Nitrite accumulation in the medium (a) was determined using the Griess reaction and intracellular citrulline concentrations by reversed-phase h.p.l.c. (b). Values denote the mean ± s.e. of measurements in 4 separate experiments. *$P<0.05$ Student's unpaired t test.

Discussion

The present results demonstrate that LPS induces arginine transport activity, in addition to NO synthase, in the murine macrophage cell line J774. More importantly, our findings show that although induction of both these processes depends on *de novo* protein synthesis, each is regulated differentially by glucocorticoids. Activation of cells by LPS in the presence of dexamethasone resulted in a complete inhibition of nitrite and citrulline production, whereas induction of arginine transport and changes in intracellular arginine concentrations were unaffected. Moreover, addition of IFN-γ further increased LPS-stimulated nitrite production (Figure 2), but has no additional effect on arginine transport (Bogle *et al.*, 1992a).

Uptake of arginine in macrophages occurs via a carrier-mediated system which has been identified as y^+ (Bogle *et al.*, 1992a; Sato *et al.*, 1992). System y^+ operates as a facilitated diffusion carrier which accumulates arginine against a substantial concentration gradient (~10 fold in J774 cells, see Tables 1 and 2). It is driven by the membrane potential (Bussolati *et al.*, 1987) and in macrophages, as in other cell types, is sensitive to *trans*-stimulation (White, 1985; Sato *et al.*, 1991). These two mechanisms, in addition to synthesis of new carrier protein, could in principle contribute to the enhanced rates of arginine uptake in activated cells. As LPS either has no effect on or depolarizes the membrane potential (Larsen *et al.*, 1985; Henry-Toulme *et al.*, 1990) and amino acid levels are decreased in activated J774 cells (Tables 1 and 3), these effects would reduce rather than enhance uptake of arginine in LPS-stimulated macrophages.

Induction of NO synthase in macrophages is strongly inhibited by glucocorticoids such as dexamethasone and hydrocortisone (Di Rosa *et al.*, 1990), probably acting at the level of transcription of mRNA. We confirmed that dexamethasone substantially inhibited nitrite and citrulline production in LPS-activated J774 cells (Figure 2). Interestingly, the synergistic actions of LPS/IFN-γ on nitrite and citrulline production were only partially inhibited by dexamethasone. It has recently been shown that LPS/IFN-γ act synergistically at the level of transcription of NO synthase mRNA (Lors-

Table 3 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on total amino acid concentrations in J774 cells and culture medium

	Total amino acid concentration (mM)		
Condition	*J774 cells*	*Medium*	*Cell/Medium*
Control	86.4 ± 9.9	12.7 ± 0.3	6.8
LPS (1 μg ml^{-1})	58.2 ± 4.0*	15.7 ± 1.4	3.7
IFN-γ (100 units ml^{-1})	48.7 ± 6.0*	16.4 ± 1.3	3.0
LPS + IFN-γ (100 units ml^{-1})	32.5 ± 2.6*	16.4 ± 0.8	2.0
LPS + dexamethasone (1 μM)	60.3 ± 2.6*	13.2 ± 1.3	4.6
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	28.1 ± 2.7*	14.7 ± 1.7	1.9

Details as in legends to Tables 1 and 2. Cell to medium ratios were estimated from the mean amino acid concentrations. All other values denote the mean ± s.e. of measurements in 4 experiments. *$P<0.05$, Student's unpaired t test.

bach *et al.*, 1993). Our result therefore indicates that transcriptional modulation by dexamethasone may be less effective when IFN-γ-induced promoters are present.

Induction of arginine transport by LPS was unaffected by treatment of J774 cells with a concentration of dexamethasone that abolished nitrite production. This observation suggests that the system y^+ gene is not sensitive to glucocorticoids. Dexamethasone and other natural steroids have been reported to enhance hormone-stimulated transport of neutral amino acids (see review, Guidotti *et al.*, 1978), but we are unaware of any studies reporting the effects of dexamethasone on system y^+. Our results also show that production of NO does not serve as an autocrine loop to induce arginine transport. We have previously shown that arginine uptake by endothelial cells is rapidly and transiently upregulated during agonist-stimulated release of NO via constitutive NO synthase; however, this was also predominantly independent of NO production (Bogle *et al.*, 1991).

There is controversy as to whether citrulline generated by NO synthase in activated macrophages can be recycled to arginine, as reported intially in endothelial cells (Hecker *et al.*, 1990). Although LPS-activated rat peritoneal macrophages apparently recycle citrulline to arginine (Wu & Brosnan, 1992), studies with murine peritoneal macrophages suggest that citrulline only accumulates in the extracellular medium (Benninghoff *et al.*, 1991). Our current experiments have shown that citrulline accumulates both intracellularly and in the culture medium of activated J774 cells, and in preliminary experiments we have found that J774 cells transport and metabolise citrulline to arginine which can then be used for NO synthesis (Bogle *et al.*, 1992b).

In activated J774 cells, transported arginine may be metabolised via pathways other than inducible NO synthase. In activated murine macrophages only 30% of cellular L-arginine metabolism occurs via NO synthase (Granger *et al.*, 1990), though there is disagreement as to whether the flux of arginine to ornithine via arginase is concomitantly enhanced in activated macrophages (Benninghoff *et al.*, 1991; Sato *et al.*, 1992). We found increases in intracellular ornithine in J774 cells generating nitrite (Table 1) with negligible changes in extracellular ornithine (Table 2). Studies with selective inhibitors of ornithine decarboxylase, the main metabolic route for ornithine metabolism leading to polyamine synthesis, could be designed to test the importance of this pathway in the induction of system y^+. However, we believe it is more likely that induction of arginine transport by LPS is regulated independently of the intracellular metabolism of arginine. Investigation of the regulatory sequence of the system y^+ gene, recently identified (Kim *et al.*, 1991; Wang *et al.*, 1991), will be needed to substantiate this hypothesis.

Finally, it should be noted that the K_m of arginine for NO synthase ($<10\,\mu M$) is much lower than for arginase (6.8–18 mM; Kaysen & Strecker, 1973), and thus alterations in the flux of arginine via arginase should have minimal effects on the flux through NO synthase. In addition, we wish to emphasize that despite a fall of about 50% in the intracellular arginine pool in LPS/IFN-γ-activated J774 cells synthesizing NO, this pool size is theoretically well in excess of the amount required for maximal rates of NO production. Nonetheless, the rate of NO synthesis in these cells is directly related to the rate of entry of exogenous arginine (Bogle *et al.*, 1992a; Assreury & Moncada, 1992). We conclude that extracellular arginine is preferentially used to generate NO in activated macrophages, and that the intracellular pathways mediating induction of transport and NO synthase are regulated differentially by dexamethasone.

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