

Discrimination between citrulline and arginine transport in activated murine macrophages: inefficient synthesis of NO from recycling of citrulline to arginine

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1 The kinetics, specificity, pH- and Na⁺-dependency of L-citrulline transport were examined in unstimulated and lipopolysaccharide (LPS)-activated murine macrophage J774 cells. The dependency of nitric oxide production on extracellular arginine or citrulline was investigated in cells activated with LPS (1 µg ml⁻¹) for 24 h.

2 In unstimulated J774 cells, transport of citrulline was saturable ($K_i = 0.16$ mM and $V_{max} = 32$ pmol µg⁻¹ protein min⁻¹), pH-insensitive and partially Na⁺-dependent. In contrast to arginine, transport of citrulline was unchanged in LPS-activated (1 µg ml⁻¹, 24 h) cells.

3 Kinetic inhibition experiments revealed that arginine was a relatively poor inhibitor of citrulline transport, whilst citrulline was a more potent inhibitor ($K_i = 3.4$ mM) of arginine transport but only in the presence of extracellular Na⁺. Neutral amino acids inhibited citrulline transport ($K_i = 0.2$ – 0.3 mM), but were poor inhibitors of arginine transport.

4 Activated J774 cells did not release nitrite in the absence of exogenous arginine. Addition of citrulline (0.01–10 mM), in the absence of exogenous arginine, could only partially restore the ability of cells to synthesize nitrite, which was abolished by 100 µM N^G-nitro-L-arginine methyl ester or N^G-iminoethyl-L-ornithine.

5 Intracellular metabolism of L-[¹⁴C]-citrulline to L-[¹⁴C]-arginine was detected in unstimulated J774 cells and was increased further in cells activated with LPS and interferon-γ.

6 We conclude that J774 macrophage cells transport citrulline via a saturable but nonselective neutral carrier which is insensitive to induction by LPS. In contrast, transport of arginine via the cationic amino acid system y⁺ is induced in J774 cells activated with LPS.

7 Our findings also confirm that citrulline can be recycled to arginine in activated J774 macrophage cells. Although this pathway provides a mechanism for enhanced arginine generation required for NO production under conditions of limited arginine availability, it cannot sustain maximal rates of NO synthesis.

Keywords: Murine macrophage cell line J774; macrophages; nitric oxide; nitrite; citrulline transport; arginine transport; citrulline metabolism; bacterial lipopolysaccharide

Introduction

Synthesis and release of nitric oxide (NO) by activated macrophages is an important cytotoxic/cytostatic mechanism in non-specific immunity (see review by Moncada *et al.*, 1991). In macrophages, NO and citrulline are generated from arginine by a Ca²⁺/calmodulin-insensitive NO synthase, which is induced following exposure of these cells to lipopolysaccharide (LPS) and/or cytokines such as interferon-γ (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Bogle *et al.*, 1992a). Production of NO by activated macrophages is critically dependent on extracellular arginine (Drapier & Hibbs, 1988; Granger *et al.*, 1990; Keller *et al.*, 1990; Takema *et al.*, 1991; Bogle *et al.*, 1992a; Assreuy & Moncada, 1992).

Recent studies in macrophages have demonstrated that arginine (Bogle *et al.*, 1992a; Sato *et al.*, 1992) and lysine (Sato *et al.*, 1991) are transported by a high-affinity carrier, resembling the cationic amino acid system y⁺ identified in a variety of other cell types (White, 1985). We have reported that induction of arginine transport activity in LPS-stimulated J774 macrophage cells involves *de novo* protein synthesis, suggesting that enhanced uptake of arginine is important for sustained NO biosynthesis (Baydoun *et al.*, 1993). In view of

the fact that peritoneal macrophages can recycle citrulline to arginine (Wu & Brosnan, 1992), we were interested in establishing whether this pathway could sustain NO production in LPS-activated J774 cells. We have therefore investigated the ability of J774 cells to convert citrulline to arginine, and examined whether this generates sufficient arginine for sustained NO production under conditions of limited arginine availability. In addition, we have fully characterized citrulline transport in unstimulated and LPS-activated J774 cells with the aim of determining whether separate pathways mediate the entry of citrulline and arginine. A preliminary account of this work has been presented in abstract form (Bogle *et al.*, 1992b).

Methods

Cell culture

The murine monocyte/macrophage cell line J774 was obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire). J774 cells were maintained in continuous culture in T75 tissue culture flasks in Dulbecco's modified Eagles medium (DMEM) containing 0.4 mM L-arginine and no L-citrulline but supplemented with 4 mM glutamine, penicillin (100 units ml⁻¹), streptomycin (100 µg ml⁻¹) and 10% foetal calf serum (providing a further

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0.08 mM L-arginine and 0.01 mM L-citrulline). Cells were harvested by gentle scraping and passed every 3–6 days by dilution of a suspension of the cells 1:10 in fresh medium.

Measurement of amino acid 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. Medium was then replaced either with fresh DMEM or with DMEM containing LPS ($1 \mu\text{g ml}^{-1}$) and/or other compounds for specific time periods. After each incubation, cells were rinsed twice with a modified HEPES-buffered Krebs solution maintained at 37°C (Bogle *et al.*, 1992a). Amino acid uptake was initiated by adding HEPES-buffered Krebs ($50 \mu\text{l}$ per well; 37°C) containing either $0.1 \text{ mM L-[}^{14}\text{C]-citrulline}$ ($1 \mu\text{Ci ml}^{-1}$) or $0.1 \text{ mM L-[}^3\text{H]-arginine}$ ($1 \mu\text{Ci ml}^{-1}$) to the monolayers. Incubations were terminated by placing the plates on melting ice and rinsing cells three times with $200 \mu\text{l}$ ice-cold Dulbecco's phosphate-buffered saline (mM: NaCl 138, KCl 2.6, Na_2HPO_4 1.8, KH_2PO_4 1.5, pH 7.4) containing either 10 mM unlabelled citrulline or arginine to remove extracellular radiolabelled amino acids. In some experiments an extracellular reference tracer, either D-[^3H]-mannitol or D-[^{14}C]-mannitol, was included in the incubation medium. In all experiments $<0.01\%$ of D-mannitol applied was recovered in cell lysates. Cell protein was determined using the BioRad reagent and radioactivity (d.p.m.) in formic acid digests of the cells was determined by liquid scintillation counting. Uptake was then calculated and expressed in units of $\text{pmol } \mu\text{g}^{-1} \text{ protein min}^{-1}$.

Metabolism of L-citrulline to L-arginine

Metabolism of L-[^{14}C]-citrulline was assessed by thin-layer chromatography (t.l.c.). J774 cells were seeded into 35 mm dishes (3×10^6 cells) and allowed to adhere for 2 h. Thereafter, the medium was removed and replaced with DMEM containing $1 \mu\text{Ci ml}^{-1}$ L-[^{14}C]-citrulline alone or supplemented with LPS ($1 \mu\text{g ml}^{-1}$) and IFN- γ ($100 \text{ units ml}^{-1}$). Following a 24 h incubation period, the medium was collected and centrifuged at $10,000 g$ for 5 min. Cells were washed with ice-cold phosphate buffered saline and lysed with 100% methanol. Medium and methanol cell lysate samples were stored at -70°C for chromatographic analysis.

Methanol extracts were evaporated to dryness under a stream of nitrogen and resuspended in $20 \mu\text{l}$ methanol of which $10 \mu\text{l}$ was spotted onto silica-coated t.l.c. plates (Whatman, 150A). For analysis of culture medium, $20 \mu\text{l}$ of deproteinized sample was spotted onto t.l.c. plates. The plates were developed in a solvent system of chloroform:methanol:ammonium hydroxide:water (5:45:20:10 v/v) over a distance of 18 cm (Iynengar *et al.*, 1987). After drying, plates were scanned with a Berthold t.l.c. linear analyzer (Berthold, Germany). The R_F -values for ^{14}C -labelled L-arginine and L-citrulline (0.32 and 0.87) were identical to those of authentic amino acid standards.

Measurement of nitrite formation

Production of NO in intact J774 cells was assessed as described previously (Bogle *et al.*, 1992a) by measuring the accumulation of nitrite in the medium using the Griess reaction with sodium nitrite as standard (Green *et al.*, 1982).

Materials

The HEPES-buffered Krebs solution was 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 . In some experiments the standard Krebs solution was titrated with 0.1 N HCl or 0.1 N NaOH to achieve pH values ranging pH 5 to pH 8. In sodium-free experiments, the buffer was modified by replacing NaCl, NaHCO_3 and NaH_2PO_4

with choline chloride, choline bicarbonate and KH_2PO_4 , respectively.

All reagents for cell culture except foetal calf serum (Sigma) were from Gibco (Paisley, U.K.). Arginine-free DMEM was prepared in the laboratory and was supplemented with 10% dialysed foetal calf serum. Serum was dialysed twice for 48 h at 4°C using a membrane with a 10,000 M_r cut off and confirmed to be free of amino acids by high performance liquid chromatography (h.p.l.c.) (Baydoun *et al.*, 1993). LPS extracted from *Escherichia coli* (serotype 055:B5) was obtained from Difco, Michigan, U.S.A. Recombinant murine IFN- γ was from Holland Biotechnologies. Other chemicals were from Sigma or BDH and of the highest grade obtainable. Radioactive tracers, L-[2,3- ^3H]-arginine (53 Ci mmol^{-1}) and L-[carbamoyl- ^{14}C]-citrulline ($54.3 \text{ mCi mmol}^{-1}$) were obtained from New England Nuclear, Dreieich, Germany. Purity of L-[^{14}C]-citrulline was $>99\%$ as assessed by t.l.c.

Statistics

All values are means \pm s.e. of at least three separate experiments with six replicates per experiment. Statistical analyses were performed by Student's *t* test with $P < 0.05$ considered statistically significant.

Results

Characteristics of citrulline transport

Time course experiments revealed that transport of $0.1 \text{ mM L-[}^{14}\text{C]-citrulline}$ was linear for up to 1 min but lower in LPS-activated cells over prolonged periods (inset Figure 1). In unstimulated J774 cells transport of citrulline was temperature-dependent and unaffected by changes in extracellular pH, ranging from 5–8 (data not shown). In contrast to arginine, citrulline transport was partially dependent on extracellular Na^+ and was unaltered by LPS (Table 1).

Transport of citrulline was saturable and a single Michaelis-Menten entry site analysis revealed an apparent K_t of $0.16 \pm 0.02 \text{ mM}$ and V_{max} of $32 \pm 1 \text{ pmol } \mu\text{g}^{-1} \text{ protein min}^{-1}$ in control J774 cells (Figure 1). Activation of cells with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h had no significant effect on the kinetics of citrulline transport ($K_t = 0.11 \pm 0.02 \text{ mM}$ and $V_{\text{max}} = 31 \pm$

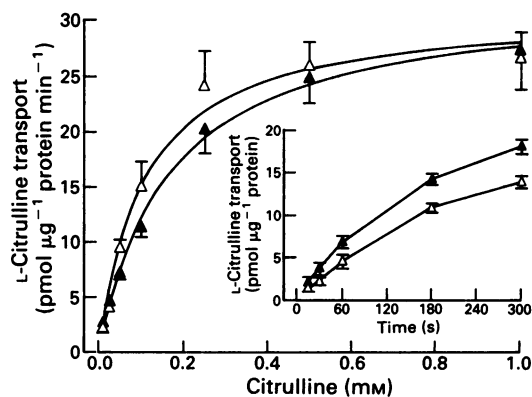


Figure 1 Kinetics and time course of citrulline uptake in unstimulated and LPS-activated J774 cells. Initial uptake rate kinetics were measured for citrulline (0.025 – 1 mM) in unstimulated (\blacktriangle) and LPS-activated (\triangle , $1 \mu\text{g ml}^{-1}$ for 24 h) cells. Rectangular hyperbolae were fitted to the mean influx data weighted for the reciprocal of the standard error at each mean. Values are the mean \pm s.e. of 6 replicate measurements in 3 separate experiments. The inset shows the time course of L-[^{14}C]-citrulline transport (0.1 mM) measured in unstimulated cells (\blacktriangle) or cells pretreated with LPS (\triangle , $1 \mu\text{g ml}^{-1}$ for 24 h) over 15–300 s incubation periods. Values are the mean \pm s.e. of 6 replicate measurements in 3 different experiments.

Table 1 Sodium-dependency of L-citrulline and L-arginine transport in J774 cells

	Unstimulated J774 cells (pmol μg^{-1} protein min^{-1})		+ LPS (1 $\mu\text{g ml}^{-1}$) (pmol μg^{-1} protein min^{-1})	
	+ Na ⁺	- Na ⁺	+ Na ⁺	- Na ⁺
L-Citrulline	11.7 \pm 0.6	6.8 \pm 0.3*	11.6 \pm 0.9	7.8 \pm 0.4*
L-Arginine	4.0 \pm 0.1	3.8 \pm 0.1	6.5 \pm 0.2	6.0 \pm 0.2

Transport of L-[¹⁴C]-citrulline (0.1 mM) or L-[³H]-arginine (0.1 mM) was measured over 30 s and 5 min, respectively, during incubation of J774 cells in a Krebs solution containing 143 or 0 mM Na⁺. Cells were activated with LPS for 24 h in DMEM before assessing the Na⁺-dependency of amino acid transport during incubation with Krebs. Values are the mean \pm s.e. of 12–18 replicate measurements in 3 separate experiments.

* $P < 0.05$, unpaired *t* test versus values in the presence of Na⁺.

1 pmol μg^{-1} protein min^{-1}). The insensitivity of citrulline transport to LPS contrasts markedly with our earlier findings that the V_{max} for arginine transport was increased in LPS-activated J774 cells (Bogle *et al.*, 1992a).

Selectivity of citrulline transport

In kinetic inhibition experiments, both citrulline and arginine caused a marked self-inhibition of transport (Figure 2a and b). Arginine (0.5–5 mM) was a poor inhibitor of citrulline transport (Figure 2a), whereas citrulline significantly inhibited ($K_i = 3.4 \pm 0.5$ mM) arginine uptake (Figure 2b). Although arginine transport in J774 cells is Na⁺-independent (Table 1), the inhibition of arginine uptake by citrulline was abolished in the absence of extracellular Na⁺ (Figure 2b).

The substrate specificities of citrulline and arginine transporters were examined further by screening the inhibitory effects of a series of 1 mM unlabelled amino acids. As shown in Figure 3, the cationic substrates for system y⁺, arginine and lysine, and the system A analogue, 2-methylaminoisobutyric acid (MeAIB), were weak inhibitors of citrulline (0.1 mM) transport in unstimulated and LPS-activated cells. In contrast, transport was inhibited markedly by naturally occurring and synthetic neutral amino acids, and the pattern of inhibition was similar in both unstimulated and activated cells (Figure 3). Thus, in J774 macrophage cells transport of citrulline seems to be mediated by a neutral carrier with broad substrate specificity, resembling the neutral amino acid system identified by Sato *et al.* (1987) in murine peritoneal macrophages.

In contrast, transport of arginine (0.1 mM) was inhibited significantly by cationic amino acids, β -2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) and citrulline, but not by MeAIB, phenylalanine, 6-diazo-5-oxo-L-norleucine (DON) or glutamine (data not shown). No significant change in the pattern of inhibition occurred when cells were activated with LPS.

Metabolism of citrulline to arginine in cultured J774 cells

Metabolism of L-[¹⁴C]-citrulline by cultured J774 cells was investigated by thin-layer chromatography. Incubation of unstimulated J774 cells with L-[¹⁴C]-citrulline for 24 h resulted in the production of L-[¹⁴C]-arginine which was detected in both cell extracts and the culture medium (Table 2). Activation of cells with LPS (1 $\mu\text{g ml}^{-1}$) and interferon- γ (IFN- γ , 100 units ml^{-1}) for 24 h resulted in a significantly greater intracellular conversion of L-[¹⁴C]-citrulline into L-[¹⁴C]-arginine.

Effects of citrulline on nitrite production from arginine-deprived J774 cells

In a previous study we reported that nitrite release from activated J774 cells was not detectable when L-arginine was omitted from the culture medium (Bogle *et al.*, 1992a). Since citrulline has been reported to recycle to arginine intracel-

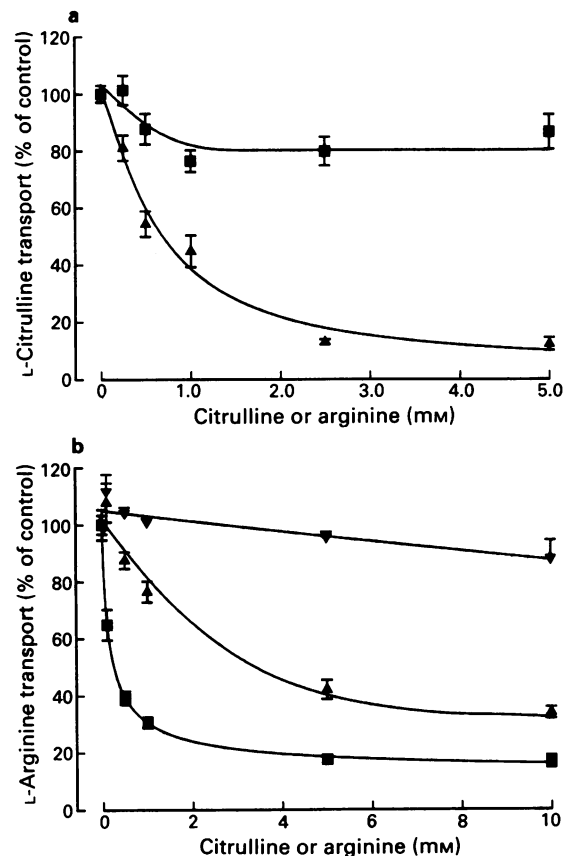


Figure 2 Discrimination of citrulline and arginine transport. (a) Inhibition of 0.1 mM L-[¹⁴C]-citrulline transport was measured in the presence of Na⁺ and increasing concentrations (0–5 mM) of unlabelled L-citrulline (\blacktriangle) or L-arginine (\blacksquare). Values are the mean \pm s.e. of 6 replicate measurements in 3 separate experiments. (b) Inhibition of 0.1 mM L-[³H]-arginine influx by increasing concentrations (0–10 mM) of unlabelled L-arginine (\blacksquare) or L-citrulline (\blacktriangle) in cells incubated in the presence of Na⁺. Inhibition of arginine transport by citrulline was abolished in the absence of extracellular Na⁺ (\blacktriangledown). Values are the mean \pm s.e. of 10 replicate measurements in 3 experiments.

ularly in both endothelial cells (Hecker *et al.*, 1990) and peritoneal macrophages (Wu & Brosnan, 1992), we designed experiments to investigate whether citrulline could restore nitrite production in cultured J774 cells activated in the absence of exogenous arginine. When activated cells were incubated in an arginine-free medium containing citrulline (0.01–10 mM), nitrite production was detectable, reaching a maximum of 2.1 ± 0.1 nmol μg^{-1} protein 24 h^{-1} in the presence of 10 mM citrulline (Figure 4). Maximal rates of nitrite production (8.1 ± 0.22 nmol μg^{-1} protein 24 h^{-1}) were achieved at extracellular arginine concentrations of less than 0.3 mM. Thus, citrulline supply can only sustain $\sim 20\%$ of

Table 2 Metabolism of L-[¹⁴C]-citrulline to L-[¹⁴C]-arginine in cultured J774 cells

	% L-[¹⁴ C]-arginine	% L-[¹⁴ C]-citrulline
<i>Unstimulated J774 cells</i>		
Culture medium	30 ± 3	
Cell extracts	17 ± 1	70 ± 3
		71 ± 2
<i>J774 cells + LPS/IFN-γ</i>		
Culture medium	24 ± 1	76 ± 1
Cell extracts	40 ± 4*	42 ± 8*

J774 cells were cultured in 35 mm dishes (3×10^6 cells) in the absence or presence of LPS ($1 \mu\text{g ml}^{-1}$) and IFN- γ ($100 \text{ units ml}^{-1}$). L-[¹⁴C]-citrulline ($1 \mu\text{Ci ml}^{-1}$) was added at time zero and 24 h later cells and media were analysed for ¹⁴C-labelled metabolites of citrulline by t.l.c. Results are shown as the % radioactivity associated with each detected peak and denote the mean \pm s.e. of 3 experiments.

* $P < 0.05$, values significantly different from control cells, Student's unpaired *t* test.

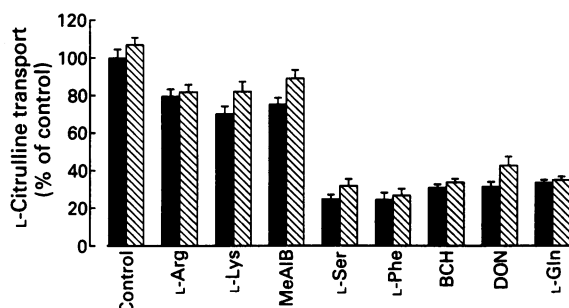


Figure 3 Specificity of citrulline transport in unstimulated and LPS-activated J774 cells. Transport of 0.1 mM L-[¹⁴C]-citrulline was measured in the absence (control) or presence of a given inhibitor amino acid (1 mM). Cross-inhibition studies were performed in unstimulated (solid columns) and lipopolysaccharide-activated ($1 \mu\text{g ml}^{-1}$, 24 h, hatched columns) cells in the presence of Na^+ . Data are expressed as a percentage of the transport rate measured in unstimulated cells in the absence of an inhibitor. Abbreviations denote standard amino acid nomenclature: arginine (Arg), lysine (Lys, system γ^+); 2-methylaminoisobutyric acid (MeAIB, system A); serine (Ser, system ASC/asc); phenylalanine (Phe), β -2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH, system L); 6-diazo-5-oxo-L-norleucine (DON), glutamine (Gln, system N). Values denote the mean \pm s.e. of 6 replicate measurements in 3 experiments.

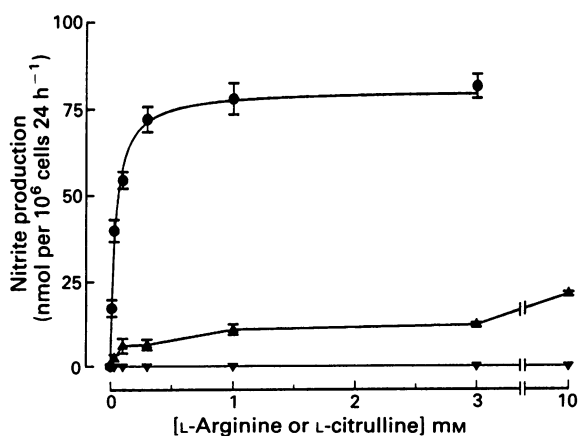


Figure 4 Dependency of nitrite production on extracellular citrulline or arginine in activated J774 cells. Cells were preincubated for 24 h with lipopolysaccharide ($1 \mu\text{g ml}^{-1}$) and interferon- γ ($100 \text{ units ml}^{-1}$) in an arginine-free medium supplemented with either L-citrulline (0.01 – 10 mM), in the absence (\blacktriangle) or presence of N^G -imidoethyl-L-ornithine (L-NIO, \blacktriangledown , 0.1 mM). Accumulation of nitrite in the medium was then assayed using the Griess reaction. The dependency of nitrite production on extracellular L-arginine (\bullet , 0 – 3 mM) is shown for comparison and was replotted from Figure 2 in Bogle *et al.* (1992a). Values are the mean \pm s.e. of 6 replicate measurements in 3 experiments.

the maximal nitrite production generated from physiological concentrations of extracellular arginine.

In arginine-deprived J774 cells nitrite production, generated from citrulline, was abolished during co-incubation of cells with either N^G -nitro-L-arginine methyl ester (L-NAME; 0.1 mM ; data not shown) or N^G -imidoethyl-L-ornithine (L-NIO; 0.1 mM ; Figure 4), both potent inhibitors of inducible NO synthase (Rees *et al.*, 1990; McCall *et al.*, 1991).

Discussion

The present study confirms that uptake of citrulline and arginine by J774 cells is mediated by different amino acid transporters: a neutral amino acid carrier with broad substrate specificity and the cationic amino acid system γ^+ . Although metabolism of citrulline to arginine occurs in J774 macrophages and is enhanced following activation of cells with LPS, citrulline can only sustain limited NO synthesis in the absence of exogenous arginine. The rate of NO production approximates only $\sim 20\%$ of the maximal rate achieved with arginine, despite comparable or greater transport rates for citrulline.

Little information is available on the mechanism(s) mediating cellular uptake of citrulline. Studies *in vivo* suggest that citrulline is synthesized and released from the intestine as an end product of glutamine nitrogen metabolism (Windmueller & Spaeth, 1981). In the circulation, citrulline is rapidly cleared by the kidney and subsequently metabolised and released as arginine (Dhanakoti *et al.*, 1990). Thus, uptake and metabolism of citrulline may contribute to the maintenance of plasma arginine levels which appear to be important for sustained NO production *in vivo* (Aisaka *et al.*, 1989).

In our studies, uptake of citrulline by J774 cells occurred against a 5 fold concentration gradient (Baydoun *et al.*, 1993), was saturable, partially Na^+ -dependent and reduced at low temperature. Moreover, the kinetics of citrulline transport were not altered in LPS-activated J774 cells. This contrasts with our previous report that arginine transport via system γ^+ was induced in activated J774 cells (Bogle *et al.*, 1992a). Recent studies have also identified another cationic amino acid transporter (mouse cationic amino acid transporter, MCAT-2), of low affinity ($K_m > 2 \text{ mM}$), in mouse hepatocytes and the murine macrophage cell line RAW264.7 (Closs *et al.*, 1993a,b). Although the MCAT-2 transporter reveals a similar sequence and structure to MCAT-1 (high affinity cationic γ^+ transporter, Kim *et al.*, 1991), only 5–10% of MCAT-2 transport would be occupied by substrate at physiological plasma concentrations. As the specificity of the arginine transporter is similar in unstimulated and activated J774 cells (Bogle *et al.*, 1992a,b; Baydoun *et al.*, 1993) and murine peritoneal macrophages (Sato *et al.*, 1992), it is likely that in these cell types the activity of system γ^+ is upregulated by LPS.

Very few studies have investigated the relationship between

citrulline and arginine transport. Inhibition of arginine transport by citrulline has been observed in the chicken small intestine (Herzberg *et al.*, 1971). In the present experiments inhibition of arginine uptake by citrulline was observed but was abolished when Na⁺ was removed from the incubation medium. This phenomenon of Na⁺-dependent inhibition of Na⁺-independent cationic amino acid transport has been observed previously in both reticulocytes and Ehrlich cells (Christensen & Antonioli, 1969). At physiological pH, citrulline carries no net charge and can therefore be classified as a neutral amino acid. It is likely that Na⁺ serves as a substitute for the positively charged side chain of arginine, thus allowing a fraction of citrulline to enter J774 cells via system y⁺. The inhibition of arginine transport by neutral amino acids in the presence of sodium is indeed a feature of system y⁺ (Munck, 1980; White, 1985; Lawless *et al.*, 1987; Sweiry *et al.*, 1991).

Although activation of J774 cells did not markedly alter the kinetics of citrulline transport, net accumulation of ¹⁴C from [¹⁴C]-citrulline over a 60 min incubation period was reduced by ~50% in LPS-treated cells (data not shown). Since in these cells the V_{max} for citrulline transport was not altered, this difference may reflect increased metabolism and subsequent efflux of either citrulline itself or a radiolabelled metabolite. In this respect we have previously shown by reverse-phased h.p.l.c. that citrulline accumulates in the culture medium of activated cells (Baydoun *et al.*, 1993). Our present results indicate that J774 cells can synthesize L-[¹⁴C]-arginine from L-[¹⁴C]-citrulline, which is increased further following activation of J774 cells with LPS and IFN-γ. These

findings suggest that the enzymatic pathway, arginosuccinate synthase/lyase (Hoffman *et al.*, 1978), responsible for citrulline metabolism is induced by treatment of J774 cells with these agents. Wu & Brosnan (1992) have reported a 3 fold increase in the metabolism of citrulline to arginine in LPS-treated rat peritoneal macrophages. Moreover, recent studies in rat smooth muscle cells and the RAW 264.7 macrophage cell line demonstrate that LPS and cytokines induce arginosuccinate synthase gene expression which appears to be essential for sustaining NO production in cells expressing inducible NO synthase (Morris *et al.*, 1993).

At sites of macrophage activation *in vivo*, rapid depletion of extracellular arginine occurs due to the metabolism of arginine by both arginase and NO synthase (Currie *et al.*, 1978; Albina *et al.*, 1989; Granger *et al.*, 1990). Under these conditions the availability of arginine may become rate-limiting for NO production. Thus, metabolism of citrulline to arginine could be an important mechanism not only for the maintenance of intracellular arginine levels but also for the production of NO. Our results, however, demonstrate that this process is at best very inefficient, emphasising the importance of an adequate supply of extracellular arginine to sustain NO synthesis in activated macrophages.

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References

- AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). L-arginine availability determines the duration of acetylcholine-induced systemic vasodilatation *in vivo*. *Biochem. Biophys. Res. Commun.*, **163**, 710–717.
- ALBINA, J.E., MILLS, C.D., HENRY, W.L. & CALDWELL, M.D. (1989). Regulation of macrophage physiology by L-arginine: role of the oxidative deiminase pathway. *J. Immunol.*, **143**, 3641–3646.
- ASSREUY, J. & MONCADA, S. (1992). A perfusion system for the long term study of macrophage activation. *Br. J. Pharmacol.*, **107**, 317–321.
- BAYDOUN, A.R., BOGLE, R.G., PEARSON, J.D. & MANN, G.E. (1993). Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells. *Br. J. Pharmacol.*, **110**, 1401–1406.
- BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D., MONCADA, S. & MANN, G.E. (1992a). L-arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.*, **284**, 15–18.
- BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D. & MANN, G.E. (1992b). Citrulline can restore the ability of activated macrophages to generate nitric oxide in the absence of extracellular arginine. *Br. J. Pharmacol.*, **107**, 286P.
- CHRISTENSEN, H.N. & ANTONIOLI, J.A. (1969). Cationic amino acid transport in the rabbit reticulocyte. Na⁺-dependent inhibition of Na⁺-independent transport. *J. Biol. Chem.*, **244**, 1497–1504.
- CLOSS, E.I., ALBRITTON, L.M., KIM, J.W. & CUNNINGHAM, J.M. (1993a). Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J. Biol. Chem.*, **268**, 7538–7544.
- CLOSS, E.I., LYONS, C.R., MITCHELL, M. & CUNNINGHAM, J.M. (1993b). Expression of cationic amino acid transporters in NO-producing macrophages. *Endothelium*, **1**, S16.
- CURRIE, G.A. (1978). Activated macrophages kill tumour cells by releasing arginase. *Nature*, **273**, 758–759.
- DHANAKOTI, S.N., BROSNAN, J.T., HERZBERG, G.R. & BROSNAN, M.E. (1990). Renal arginine synthesis: studies *in vitro* and *in vivo*. *Am. J. Physiol.*, **259**, E437–E442.
- DRAPIER, J.-C. & HIBBS, J.B. Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells in L-arginine dependent inhibition of mitochondrial iron-sulphur enzymes in the macrophage effector cells. *J. Immunol.*, **140**, 2829–2838.
- GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [¹⁵N]nitrite in biological fluids. *Anal. Biochem.*, **126**, 131–138.
- GRANGER, D.L., HIBBS, J.B. Jr., PERFECT, J.R. & DURACK, D.T. (1990). Metabolic fate of L-arginine in relation to microbistatic capability of murine macrophages. *J. Clin. Invest.*, **85**, 264–273.
- HECKER, M., SESSA, W.C., HARRIS, H.J., ANGGARD, E.E. & VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 8612–8616.
- HERZBERG, G.R., SHEERIN, H. & LERNER, J. (1971). Cationic amino acid transport in chicken small intestine. *Comp. Biochem. Physiol.*, **40A**, 229–247.
- HIBBS, J.B. Jr, TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.
- HOFFMAN, F., KREUSCH, J., MAIER, K.-P., MUNDER, P.G. & DECKER, K. (1978). The urea cycle in different types of macrophages. *Biochem. Soc. Trans.*, **6**, 990–992.
- IYENGAR, R., STUEHR, D.J. & MARLETTA, M.A. (1987). Macrophage synthesis of nitrite, nitrate and N-nitrosoamines: precursors and the role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6369–6373.
- KELLER, R., GEIGES, M. & KEIST, R. (1990). L-arginine dependent reactive nitrogen intermediates as mediators of tumour cell killing by activated macrophages. *Cancer Res.*, **50**, 1421–1425.
- KIM, J.W., CLOSS, E.I., ALBRITTON, L.M. & CUNNINGHAM, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature*, **352**, 725–728.
- LAWLESS, K., MAENZ, D. & CHEESEMAN, C. (1987). Is leucine an allosteric modulator of the lysine transporter in the intestinal basolateral membrane? *Am. J. Physiol.*, **253**, G637–G642.
- MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.
- MCCALL, T.B., FEELISCH, M., PALMER, R.M.J. & MONCADA, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide in phagocytic cells. *Br. J. Pharmacol.*, **102**, 235–238.

- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.
- MORRIS, S.M., NAKAYAMA, D.K., NUSSLER, A.K., LIU, Z., DAVIES, P., PITT, B.R., SIMMONS, R.L. & BILLIAR, T.R. (1993). Co-induction of NO synthase and argininosuccinate synthetase (AS) gene expression. Implications for regulation of NO synthesis. *Endothelium*, **1**, S9.
- MUNCK, B.G. (1980). Lysine transport across the small intestine. Stimulating and inhibitory effects of neutral amino acids. *J. Memb. Biol.*, **53**, 45–53.
- PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.
- REES, D.D., SCHULZ, R., HODSON, H.F., PALMER, R.M.J. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial cell nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.
- SATO, H., FUJIWARA, M. & BANNAI, S. (1992). Effect of lipopolysaccharide on transport and metabolism of arginine in mouse peritoneal macrophages. *J. Leuk. Biol.*, **52**, 161–164.
- SATO, H., ISHII, T., SUGITA, Y. & BANNAI, S. (1991). Induction of cationic amino acid transport activity in mouse peritoneal macrophages by lipopolysaccharide. *Biochim. Biophys. Acta*, **1069**, 46–52.
- SATO, H., WATANABE, H., ISHII, T. & BANNAI, S. (1987). Neutral amino acid transport in mouse peritoneal macrophages. *J. Biol. Chem.*, **262**, 13015–13019.
- SWEIRY, J.H., MUÑOZ, M. & MANN, G.E. (1991). Cis-inhibition and trans-stimulation of cationic amino acid transport in the perfused rat pancreas. *Am. J. Physiol.*, **261**, C506–C514.
- TAKEMA, M., INABA, K., UNO, K., KAKIHARA, K.-I., TAWARA, K. & MURAMATSU, S. (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. *J. Immunol.*, **146**, 1928–1933.
- WHITE, M.F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochem. Biophys. Acta*, **822**, 355–374.
- WINDMUELLER, H.G. & SPAETH, A.E. (1981). Source and fate of circulating citrulline. *Am. J. Physiol.*, **241**, E473–E480.
- WU, G. & BROSNAN, M.T. (1992). Macrophages can convert citrulline into arginine. *Biochem. J.*, **281**, 45–48.

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