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RESEARCH COMMUNICATION

Macrophages can convert citrulline into arginine

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Rat peritoneal macrophages were incubated in the presence of 0.05–1.0 mM-[^{14}C]citrulline. The synthesis of [^{14}C]arginine from 0.1 mM-[^{14}C]citrulline was about 300 pmol/h per 10^6 cells in macrophages from saline-injected (control) rats. Both arginine synthesis from citrulline and nitrate production (an indicator of NO generation) were increased about 3-fold in the cells from lipopolysaccharide (LPS)-treated animals. The arginine synthesis was very sensitive to extracellular citrulline concentration in the range found in plasma (0.05–0.1 mM). The rate of arginine synthesis from citrulline was inhibited by about 20% by 0.5 mM-L-glutamine in both control and LPS-treated rat cells, but was inhibited by 0.5 mM-L-arginine only in control cells. Our results demonstrate that citrulline, produced by NO synthetase, can be recycled to arginine in macrophages. The citrulline–arginine cycle may contribute to the regulation of intracellular availability of arginine and thus the prolonged production of NO by macrophages.

INTRODUCTION

Macrophages play an important role in immune responses [1]. Recent studies have discovered that macrophages, like endothelial cells [2–4], synthesize NO and citrulline from L-arginine [5–10]. This arginine-dependent production of NO has been implicated in mediating the cytotoxic actions of activated macrophages against a variety of target cells, including tumour cells [8], pathogenic fungi [11], mycobacteria [12], intracellular protozoa [13] and pancreatic β-cells [14]. As a result, the study of arginine metabolism in macrophages is important in understanding the biochemical mechanisms of the functions of these immunocytes.

Arginine can be synthesized from citrulline in the body, the kidneys being the major site [15]. In addition, a recent series of studies from Vane's laboratory have shown that arginine-depleted aortic endothelial cells are capable of converting citrulline into arginine [16,17]. These findings indicate that citrulline, produced by NO synthetase [2–4], could be recycled to arginine in endothelial cells. Vane and his associates [16] also reported that the synthesis of arginine from citrulline in endothelial cells was markedly inhibited by L-glutamine or L-arginine, pointing to a possible role for amino acids in controlling the availability of arginine and the production of NO. Indeed, the degree of inhibition by glutamine is such that one must question whether citrulline conversion into arginine could be a significant event in these cells *in vivo*. Because macrophages are also known to produce NO from arginine with associated citrulline production [5–10], the present study was designed to investigate whether citrulline can be converted into arginine in these cells.

MATERIALS AND METHODS

Chemicals

L-Citrulline, L-arginine, L-glutamine, arginase (from bovine liver), urease (from jack beans; type VI), lipopolysaccharide (LPS) (from *Escherichia coli* 0127:B8, phenol extract), BSA (essentially fatty-acid-free), sulphanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrate reductase and NADPH were obtained from Boehringer Mannheim (Montreal, Canada). L-[*ureido*-^{14}C]Citrulline was obtained from New England Nuclear (Mississauga, Canada). [^{14}C]Citrulline was purifed before use as previously described [18]. LPS solution (1 mg/ml) was freshly prepared in sterile saline (0.9% NaCl) on the day of experiment.

Animals

Male Sprague–Dawley rats (250–290 g) were used throughout this study. The animals were fed *ad libitum* on a laboratory-rat chow (Purina) and had free access to water. Rats were divided into two groups, one of which was administered intraperitoneally with LPS (1 mg/kg body wt.) [19] and the other group (control) was injected intraperitoneally with saline. At 24 h after the injection, LPS-treated rats had lost an average of 17 g body wt., whereas control rats gained 5 g on average; the animals were then killed for preparation of peritoneal macrophages.

Preparation of peritoneal macrophages

Rats were killed by cervical dislocation. Peritoneal macrophages were obtained from the animals and purifed on a Ficoll/Hypaque gradient as previously described [20]. Cells were suspended in oxygenated (O_2/CO_2, 19:1) Krebs–Ringer bicarbonate buffer (119 mM-NaCl, 4.8 mM-KCl, 2.5 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 1.2 mM-KH_2PO_4 and 25 mM-$NaHCO_3$, pH 7.4) containing 5 mM-D-glucose, 20 mM-Hepes (pH 7.4) and 1% BSA. Cell viability, as assessed by Trypan Blue exclusion, was greater than 96%.

Incubation of macrophages

Macrophages [$(2–3) \times 10^6$ cells/ml] were incubated for 15–60 min at 37 °C in 10 ml silicone-treated tubes with 1 ml of oxygenated Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.05–1.0 mM-[*ureido*-^{14}C]citrulline (0.23 μCi/ml), 5 mM-D-glucose, 20 mM-Hepes (pH 7.4) and 0.5% BSA. Addition of L-glutamine (0.5 mM), L-arginine (0.5 mM) or their combinations to the incubation medium is indicated in the Tables. Incubations

Abbreviation used: LPS, lipopolysaccharide.

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were initiated by addition of cells and terminated by addition of 200 μl of 1.5 M-$HClO_4$. Parallel incubations, in which media were acidified with $HClO_4$ before addition of cells, were run as blanks. The cell extracts were neutralized with 100 μl of 2 M-K_2CO_3, and the supernatants were used for analysis of [^{14}C]arginine. Blank radioactivities were subtracted from the sample values.

Determination of [^{14}C]arginine

[^{14}C]Arginine was measured as $^{14}CO_2$ by a coupled enzyme assay involving arginase and urease [15]. Initially, we observed high background radioactivity in the blanks using this assay protocol. This was due to the very high specific radioactivity of [^{14}C]citrulline employed in this study, and therefore it was necessary to decrease this undesired background value. We found that this high blank radioactivity was effectively decreased to about 200 d.p.m. simply by including 2 mM-L-arginine or -ornithine in the assay medium. This could be due to a low reactivity of arginase with citrulline in the absence of arginine, but the mechanism involved in unknown. Therefore the assay medium used for determining [^{14}C]arginine in the present study consisted of 0.21 ml of 150 mM-phosphate buffer (pH 7.5), 0.2 ml of 10 mM-L-arginine, 0.5 ml of neutralized samples, 65 μl of urease (216 units/ml) and 25 μl of arginase (210 units/ml). The mixture was incubated at 37 °C for 1 h in 10 ml silicone-treated tubes suspended with centre wells containing 0.3 ml of NCS (Amersham, Oakville, Canada). The medium was then acidified with 200 μl of 1.5 M-$HClO_4$ and further incubated for 1 h for collecting $^{14}CO_2$. Radioactivity of $^{14}CO_2$ was measured in an LKB 1214 Rackbeta liquid-scintillation counter. The determination of [^{14}C]arginine as $^{14}CO_2$ was verified by measuring [^{14}C]arginine in the arginine fraction collected from a Beckman ion-exchange amino acid analyser [21].

Determination of nitrite and nitrate

This was done by a modification of the method of Egami & Taniguchi [22]. For determination of nitrite, neutralized samples (0.5 ml) plus 1.5 ml of water were mixed with 100 μl of sulphanilamide reagent [22], followed by addition of 100 μl of *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [22]. After standing at room temperature for 10 min, the A_{543} of the solution was measured. Nitrate was enzymically converted into nitrite by incubating 0.5 ml of samples plus 415 μl of water with 75 μl of NADPH (0.5 mg/ml) and 10 μl of nitrate reductase (5 units/ml) at 25 °C for 2 h. The conversion of nitrate into nitrite was 94–95% under the experimental conditions used.

Statistical analyses

Data were analysed by the unpaired *t* test, the paired *t* test, or one-way analysis of variance [23] as indicated in the Tables. *P* values of less than 0.05 were taken to indicate statistical significance.

RESULTS

Arginine synthesis from citrulline

The synthesis of [^{14}C]arginine from [^{14}C]citrulline in incubated macrophages was linear up to 60 min of incubation (Fig. 1) and with cell concentrations up to 3×10^6 cells/ml (results not shown).

Table 1. Synthesis of [^{14}C]arginine from [^{14}C]citrulline in macrophages

Macrophages were incubated in the presence of 0.1 mM-[^{14}C]citrulline as described in the text. An equal portion of cells from the same rat was incubated with or without 0.5 mM-L-glutamine or -L-arginine as indicated. Data are means ± S.E.M., $n = 8$ for each group: *$P < 0.01$, significantly different from the non-addition group as analysed by paired *t* test; †$P < 0.01$, significantly different from the saline-injected group as analysed by unpaired *t* test.

Addition to media	Rats...	Arginine synthesis (pmol/h per 10^6 cells)	
		Saline-injected	LPS-treated
None		285 ± 23	887 ± 120†
0.5 mM-L-Arginine		189 ± 19*	844 ± 111†
0.5 mM-L-Glutamine		215 ± 19*	724 ± 97*†
0.5 mM-L-Arginine +0.5 mM-L-glutamine		195 ± 28*	644 ± 62*†

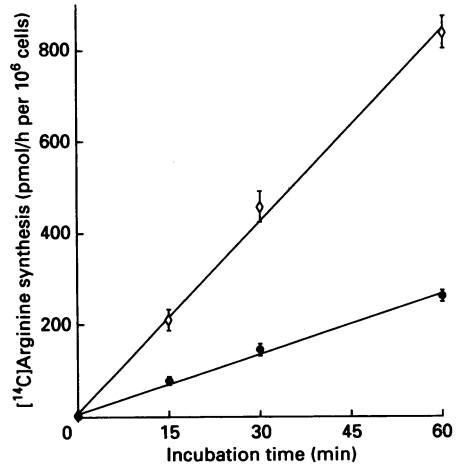

Fig. 1. Time course of [^{14}C]arginine synthesis from [^{14}C]citrulline in rat macrophages

Macrophages were incubated for 15–60 min in the presence of 0.1 mM-[^{14}C]citrulline as described in the text. Data represent means ± S.E.M., $n = 4$ for each group. Values for the control (saline) group were significantly different ($P < 0.01$) from those for the LPS-treated group at each time point as analysed by the unpaired *t* test. Key: ●, saline (control); ◇, LPS.

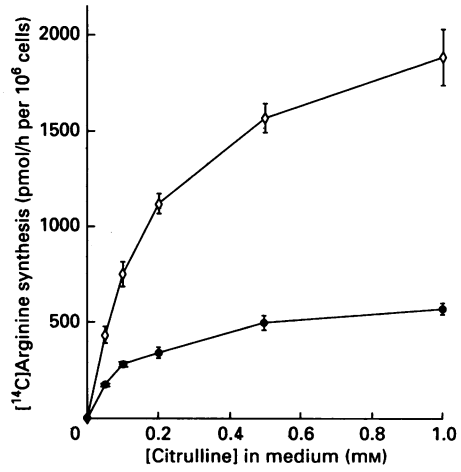

Fig. 2. [^{14}C]Arginine synthesis from [^{14}C]citrulline in rat macrophages at various extracellular citrulline concentrations

Macrophages were incubated for 1 h in the presence of 0.05–1.0 mM-[^{14}C]citrulline plus 0.5 mM-glutamine as described in the text. Data represent means ± S.E.M., $n = 4$ for each group. All mean values within each group are significantly different ($P < 0.05$) from each other as analysed by one-way analysis of variance. Key: ●, saline (control); ◇, LPS.

Table 2. Production of nitrate from L-arginine in macrophages

Macrophages were incubated in the presence of 0.1 mM-citrulline as described in the text. An equal portion of cells was incubated with or without 0.5 mM-L-arginine ± 0.5 mM-L-glutamine as indicated. Data are means ± S.E.M., $n = 8$ for each group: †$P < 0.01$, significantly different from the saline-injected group as analysed by unpaired t test. Abbreviation: ND, not detectable.

Addition to media	Rats...	Nitrate production (nmol/h per 10^6 cells)	
		Saline-injected	LPS-treated
None		ND	ND
0.5 mM-L-Arginine		1.24 ± 0.16	3.81 ± 0.54†
0.5 mM-L-Arginine + 0.5 mM-L-glutamine		1.19 ± 0.21	3.92 ± 0.49†

The rate of arginine synthesis was 3-fold greater ($P < 0.01$) in macrophages from LPS-treated rats than in the cells from control animals (Fig. 1). Addition of 0.5 mM-L-arginine or -L-glutamine inhibited ($P < 0.01$) [^{14}C]arginine synthesis by 33% and 25% respectively in unstimulated cells, with no further inhibition by arginine plus glutamine (Table 1). Interestingly, in stimulated macrophages, although 0.5 mM-L-glutamine inhibited ($P < 0.01$) this rate by 18%, 0.5 mM-L-arginine had no significant effect ($P > 0.05$) on [^{14}C]arginine synthesis from [^{14}C]citrulline (Table 1).

The rate of [^{14}C]arginine synthesis from [^{14}C]citrulline was increased with increasing citrulline concentration in the medium from 0.05 to 1.0 mM in both control and LPS-treated rat macrophages (Fig. 2). Arginine synthesis from citrulline was therefore very sensitive to citrulline concentration in the range found in rat plasma (0.05–0.1 mM) [15].

Production of nitrate from arginine

Nitrite production was undetectable in both control and LPS-treated rat macrophages incubated in either the presence or the absence of 0.5 mM-L-arginine. Likewise, we failed to detect nitrate in macrophages incubated in the absence of L-arginine. However, nitrate production was measurable when macrophages were incubated in the presence of 0.5 mM-L-arginine (Table 2). The production of nitrate by LPS-treated rat macrophages was 3-fold greater than that by control cells (Table 2). It is noteworthy that 0.5 mM-L-glutamine had no effect ($P > 0.05$) on nitrate production in either control or LPS-treated rat macrophages (Table 2).

DISCUSSION

We have demonstrated for the first time that arginine can be synthesized from citrulline in macrophages (Table 1), as previously shown for endothelial cells [16,17]. The synthesis of arginine from citrulline in both control and LPS-treated rat macrophages was not saturated even at 0.5–1.0 mM-citrulline (about 10 times its normal plasma concentration) (Fig. 2), as has been reported for renal arginine synthesis [15]. These results suggest appreciable capacity of macrophages to recycle citrulline, produced by NO synthetase, to arginine. The pathway for arginine synthesis from citrulline probably involves argininosuccinate synthetase and argininosuccinate lyase. It is noteworthy that both enzymes were previously reported to be present in macrophages [24].

It is well documented that activated macrophages increase production of NO and citrulline from L-arginine by NO synthetase [5–10]. Increased production of NO by macrophages from LPS-treated rats was also demonstrated in this study, as indicated by increased nitrate production (Table 2). It might be expected that the rate of recycling of citrulline to arginine may be increased with increasing citrulline generation in activated macrophages. This was borne out by our findings that synthesis of arginine from citrulline was 3-fold greater in LPS-treated rat macrophages than in control cells (Table 1). Since 1 mol of nitrate/nitrate production is stoichiometrically associated with 1 mol of citrulline generation from arginine in macrophages [25], it can be estimated that about 20% of arginine-derived citrulline may be recycled to arginine in resting and LPS-treated rat macophages. This may be an underestimate, as it is based on the rate of arginine synthesis from external citrulline, and it is possible that intracellularly generated citrulline may be more rapidly converted into arginine.

An arginine–citrulline cycle therefore exists in both macrophages and endothelial cells. However, it is noteworthy that there appear to be quantitative differences in the regulation of arginine synthesis from citrulline by glutamine and arginine between these two cell types. First, in contrast with endothelial cells, in which L-glutamine (even at 0.2 mM) inhibited arginine synthesis from citrulline by 82% [16], L-glutamine (0.5 mM) decreased arginine synthesis only by 25% and 18% in control and LPS-treated rat macrophages respectively (Table 1). Second, a lack of citrulline conversion into arginine was reported in arginine-sufficient endothelial cells [17], whereas the presence of 0.5 mM-L-arginine inhibited arginine synthesis only by 35% in unstimulated macrophages and had no significant effect in activated cells (Table 1). Furthermore, L-glutamine (0.1–2 mM) markedly inhibited NO production in endothelial cells [16], but had no effect in rat macrophages as indicated by nitrate production (Table 2). Although the biochemical mechanisms for the control of arginine synthesis by glutamine or arginine are unknown in either macrophages or endothelial cells, our results indicate quantitative differences in this aspect between these two NO-producing cell types.

The recycling of citrulline to arginine in macrophages may be of physiological importance. Although dietary arginine is an obvious source of arginine for metabolic utilization, endogenous production of arginine nevertheless may play some significant role in providing arginine under conditions associated with decreased food intake and increased arginine utilization. Also, since the extracellular space of inflammatory sites usually contains an exceedingly low concentration of arginine, owing to the activity of macrophage-derived arginase [26], the functions of macrophages in conditions of such a decreased extracellular arginine availability would depend, to some extent, on endogenous synthesis of arginine from citrulline. It can be speculated that this synthesis of arginine from citrulline may help macrophages to scavenge the carbon and α-amino group of L-arginine and to maintain its sufficient cellular concentration during the prolonged synthesis of NO. This may be of particular significance in activated macrophages, where NO production is markedly increased [5–10]. Thus the citrulline–arginine cycle may contribute to the regulation of intracellular availability of L-arginine, and in turn to the production of NO by macrophages.

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