Metabolism of agmatine in macrophages: modulation by lipopolysaccharide and inhibitory cytokines

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Agmatine is an amine derived from the decarboxylation of arginine by arginine decarboxylase (ADC) and metabolized to putrescine by agmatinase. While prevalent in bacteria and plants, agmatine and its metabolic enzymes have been recently identified in mammalian tissues. In the present study we sought to determine: (a) whether macrophages (cell line RAW 264.7) express ADC and agmatinase, and (b) if the enzymes are regulated by lipopolysaccharide (LPS), and/or by the inhibitory cytokines transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and interleukin-4 (IL-4). LPS induced a dose-dependent stimulation of agmatinase, while it decreased ADC, the effect in both cases being maximum at 20 h. As expected, LPS dose-dependently stimulated the inducible nitric oxide synthase activity (iNOS). A strong correlation was observed between the effects of LPS on the agmatine-related enzymes and iNOS. By contrast, exposure to IL-10 and TGF-β caused a reduction in ADC and agmatinase, whereas IL-4 was ineffective on ADC, but reverted the LPS-induced increase of agmatinase. We conclude that the agmatine pathway may be an alternative metabolic route for arginine in macrophages, suggesting a regulatory role of agmatine during inflammation.

INTRODUCTION

Agmatine is an amine formed in plants, bacteria, and some other lower lifeforms by decarboxylation of L-arginine by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19). In these organisms, agmatine is hydrolysed by agmatine urohydrolase (agmatinase; EC 3.5.3.11) to putrescine, and hence agmatine is a metabolic precursor for the biosynthesis of higher polyamines. Recently agmatine, and its metabolic enzyme ADC and agmatinase, have been detected in mammals. Agmatine and ADC are present in rat brain [1–3], kidney [4] and several cell types, including astrocytes [5], endothelium and vascular smooth-muscle cells [6], and agmatinase activity has been detected in rat brain [7]. Agmatine has been shown to act as a secretogogue [1,8,9], and possibly as a neurotransmitter/modulator in brain [10]. Agmatine has also been shown to inhibit all isoforms of nitric oxide synthases (NOSs) *in vitro* [11]. An earlier study on the metabolism of arginine in murine macrophages reported an increased decarboxylation of arginine in activated macrophages [12], but they have not addressed the specific issue of the expression of ADC activity in these cells. These facts raise the question of whether agmatine may be formed and/or degraded in macrophages, cells in which the generation of an inducible form of NOS (iNOS), is critical for generating NO, a principal mediator of the cytotoxic and cytostatic actions of these cells.

In the present study we sought to determine if macrophages express ADC and agmatinase, and, if so, whether these enzymes are regulated in relation to the induction of iNOS in macrophages by exposure to lipopolysaccharide (LPS). Furthermore, we investigated whether suppression of LPS-induced activation of iNOS by the inhibitory cytokines interleukin (IL)-4, IL-10 and transforming growth factor-β (TGF-β) will comparably influence ADC and agmatinase activities. We report that macrophages express ADC, agmatinase, and that enzyme activities are regulated by LPS and cytokines. Agmatine may play a role in modulating the state of macrophage activation during inflammation.

MATERIALS AND METHODS

Cell cultures

The murine macrophage cell line RAW 264.7 was obtained from Dr. Carl Nathan, Cornell University Medical College, New York, NY, U.S.A. and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) fetal-calf serum and 1 mM glutamine. The cells were incubated at 37 °C with 5% CO_2 in humidified air. Following exposure to LPS or cytokines in DMEM with 1% fetal-calf serum, an aliquot of the medium was removed for the assay of nitrite (see below). Cells were harvested in ice-cold PBS, and the activities of ADC and agmatinase were measured in the cell membranes and cytosol fractions respectively.

Assay for nitrites

The accumulation of nitrites in the medium was used as an indicator of NOS activity and was assayed by the Griess reaction [13]. An aliquot of the medium (100 μl) was added to 100 μl of Griess reagent in a microwell plate, and the plates were read using an ELISA plate reader at 546 nm. Using a standard curve prepared from $NaNO_3$, the amount of nitrite in the medium was calculated and the results were expressed as nmol of nitrite/mg of protein.

Abbreviations used: ADC, arginine decarboxylase; (i) NOS, (inducible) nitric oxide synthase; LPS, lipopolysaccharide; IL, interleukin; TGF-β, transforming growth factor-β; DMEM, Dulbecco's modified Eagle's medium.

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Assay of agmatinase

Agmatinase activity was measured by the production of [^{14}C]urea from guanido [^{14}C]agmatine, and subsequent trapping of $^{14}CO_2$ released from [^{14}C]urea by the addition of urease [7]. The washed cell pellets were sonicated in incubation buffer (20 mM Tris/HCl/0.32 M sucrose/1 mM EGTA, pH 7.4) and centrifuged for 20 min at 27000 ***g***. Aliquots of 300 μl of the supernatant were incubated for 30 min in the presence of 1 mM agmatine, 7 μM [^{14}C]agmatine and 0.06 unit of urease. Release of $^{14}CO_2$ from L-guanido [^{14}C]agmatine was measured by trapping the $^{14}CO_2$ in filter-paper wicks saturated with benzethonium hydroxide. The reaction was stopped by the injection of 40 % trichloroacetic acid into the reaction chamber, the filters transferred to minivials containing 5 ml CytoScint cocktail (ICN Biomedicals), and counted for radioactivity by liquid-scintillation spectrometry (Beckman model LS 5801).

Assay of ADC

Activity of ADC was measured in cell membrane fractions by the method described previously [1] that is based on the release of $^{14}CO_2$ from [1-^{14}C]arginine. Briefly, cell pellets were resuspended in Tris/EDTA buffer, pH 7.4, sonicated and centrifuged at 27000 ***g*** for 20 min. The membrane pellet was washed once by sonication and re-centrifugation in Tris/HCl buffer and re-suspended in the incubation buffer. The membrane suspension (500 μl) was incubated for 1 h at 25 °C in 20 mM Tris/HCl buffer, pH 8.25, containing 1 mM $MgSO_4$, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.2 mM EDTA, 0.1 mM L-arginine and 7.28 μM L-[1-^{14}C]arginine. As in the agmatinase assay, $^{14}CO_2$ was measured by trapping the CO_2 in filter-paper wicks saturated with benzethonium hydroxide. The reaction was stopped by the injection of 40 % trichloroacetic acid into the reaction chamber, the filters transferred to Minivials containing 5 ml of CytoScint cocktail (ICN Biomedicals) and counted for radioactivity by liquid-scintillation spectrometry.

Measurement of protein

Protein concentrations were determined using the method of Bradford [14], with BSA as standard.

Analysis of data

Results are expressed as means ± S.E.M. Data were compared between experimental groups using one-way analysis of variance combined with Fisher's test. In all cases, P values < 0.05 was considered to be statistically significant.

Materials

L-Guanido[^{14}C]agmatine was from New England Nuclear Corp. (Boston, MA, U.S.A.). L-[l-^{14}C]Arginine was from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). Recombinant human TGF-β, recombinant mouse IL-4 and recombinant murine IL-10 were from R&D Systems (Minneapolis, MN, U.S.A.). Agmatine sulphate was from Research Biochemical Int. (Natick, MA, U.S.A.). LPS (*Salmonella typhimurium*) and other chemicals were purchased from Sigma Chemical Co.

RESULTS

Effect of LPS in ADC agmatinase and NOS activities in macrophages

Macrophages constitutively express ADC (25.16 ± 1.54 nmol/h per mg of protein) in membrane fraction, agmatinase (7.41 ± 0.97 nmol/h per mg of protein) in soluble fraction and low levels of NOS (1.91 ± 0.72 nmol of NO_2/h per mg of protein). The ADC/agmatinase activity ratio was about 3.5, presumably reflecting a steady-state generation of agmatine. Incubation of cells for 20 h with LPS (1 μg/ml) (Table 1) elicited a 5.7-fold elevation of NO_2 production as a result of the induction of iNOS activity. Such treatment significantly reduced the activity of ADC by 44 %, while, reciprocally, increasing agmatinase activity to approx. 180 % of control. As a consequence the ADC/agmatinase ratio was reduced to about 1, indicating a shift favouring a reduction in the accumulation of cellular agmatine.

Table 1 Effects of LPS on the activity of ADC and agmatinase in macrophages

Cells were incubated without or with LPS (1 mg/ml) for the specified time. ADC and agmatinase activities were measured in cell membrane and cytosolic fractions respectively. Values represent mean ± S.E.M. for at least three experiments. * $P < 0.01$ compared with control (untreated) cells.

	Activity (nmol/h per mg of protein)			
	ADC		Agmatinase	
Treatment (h)	Control	LPS	Control	LPS
5	31.4 ± 2.1	34.6 ± 4.3	11.5 ± 4.2	10.5 ± 4.3
15	30.2 ± 4.5	26.5 ± 3.4	12.1 ± 3.4	13.6 ± 4.2
20	32.3 ± 3.2	15.4 ± 2.5*	8.6 ± 1.5	16.8 ± 3.4*
24	25.1 ± 3.2	28.2 ± 1.8	8.7 ± 1.8	12.5 ± 2.1
41	15.6 ± 2.5	17.8 ± 1.8	9.1 ± 2.0	9.8 ± 2.2
48	8.6 ± 1.1	9.7 ± 1.4	7.5 ± 1.5	8.9 ± 2.7

The responses of ADC and agmatinase to LPS treatment were dependent upon the duration of exposure and were reversible (Table 1). ADC activity started to decrease compared with the control at 15 h, was significantly lower by 20 h, and recovered at 24 h of exposure. In contrast, agmatinase activity was elevated by 20 h, with some recovery seen at 24 and full recovery by 41 h. At longer times the activities of enzymes in control and treated cells were reduced in parallel, probably reflecting reduction of nutrients in the culture media.

The changes in the activities of the enzymes were also dose-dependent when measured at 20 h (Figure 1). While ADC and iNOS shared comparable sensitivities to LPS with the EC_{50} values of about 100 ng/ml, agmatinase was less sensitive, having an EC_{50} value of about 200 ng/ml. To establish whether the dose-related changes in the activities correlated, the activities of agmatinase or ADC were plotted as a function of NO_2 accumulation (Figure 2). The activity of ADC was negatively correlated, whereas agmatinase was positively correlated, with iNOS activity (Figure 2). The finding suggests a close relationship between induction of iNOS and the enzymes related to the metabolism of agmatine.

Effects of IL-4, IL-10, and TGF-β on basal and LPS-induced changes in ADC, agmatinase and NOS activities

The inhibitory cytokines IL-4, IL-10, and TGF-β can suppress the induction of iNOS in LPS- or cytokine-activated macrophages [13,15–17]. We investigated whether these agents also modulated basal and/or LPS-regulated activities of ADC, agmatinase and iNOS. Macrophages were incubated for 20 h with IL-4 (5 ng/ml), IL-10 (5 ng/ml) or TGF-β (2 ng/ml), in the presence or absence of LPS (100 ng/ml). The dosages used have

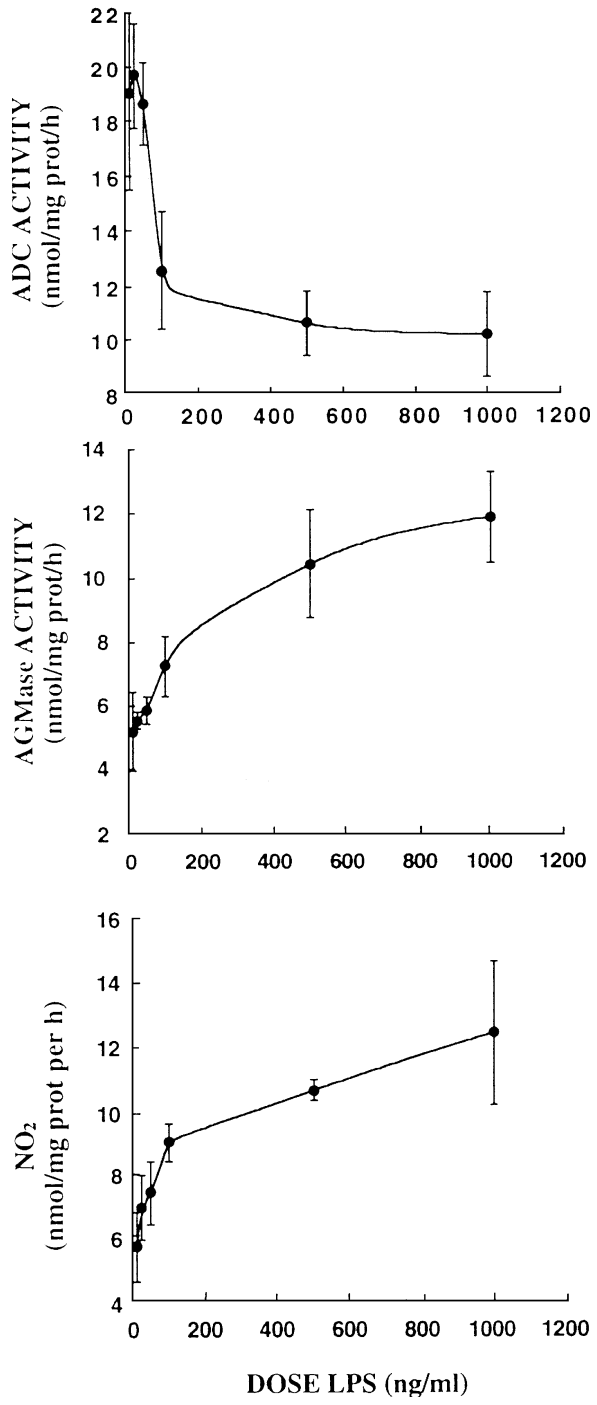

Figure 1 Effect of LPS on ADC, agmatinase and iNOS activity in macrophages

Cells were incubated for 20 h in culture medium containing various concentrations of LPS. At the end of the incubation, an aliquot of the medium was used for nitrite measurement and the harvested cells were used for the assay of agmatinase and ADC. Each point represents the mean ± S.E.M. for at least four experiments.

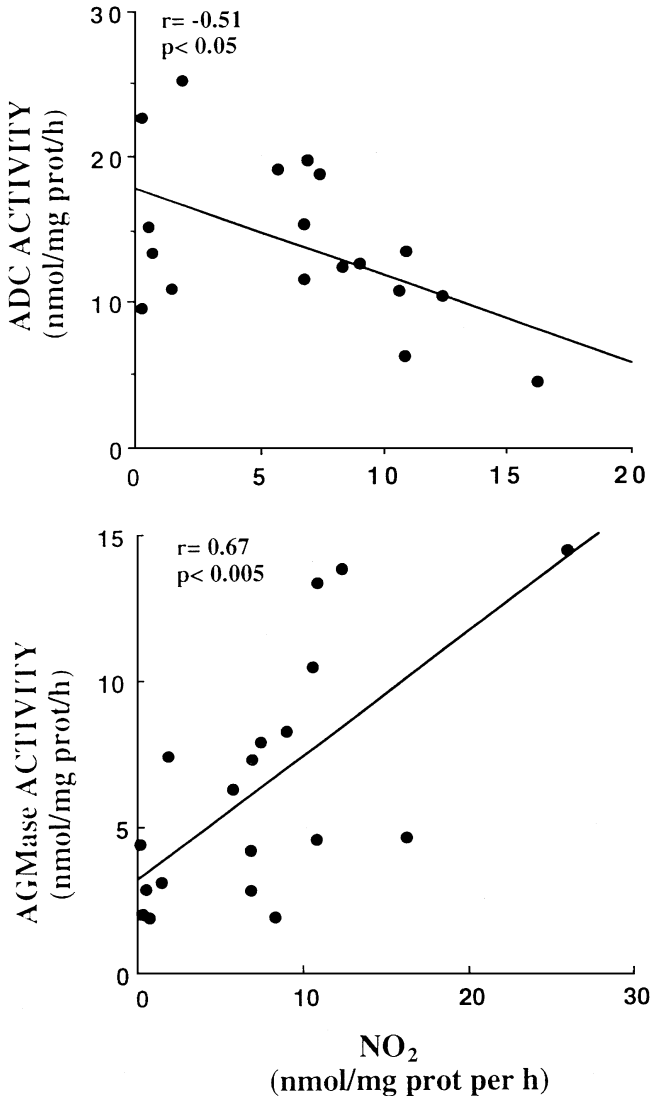

Figure 2 Correlations between agmatinase and ADC activities with NOS activity

Using data obtained from all experiments, significant positive correlation between agmatinase and NOS activities and a negative correlation between ADC and NOS activities were obtained. Lines represent regression for the correlations $y = 17.87 - 0.61x$ for ADC and $y = 3.18 + 0.42x$ for agmatinase.

been reported to have maximal effects on suppressing iNOS [16,18] in similar cells.

With one exception all three cytokines significantly reduced the basal activity of ADC, agmatinase and iNOS (Table 2). The exception was IL-4, which had no effect on the basal activity of ADC. In general the effects of the cytokines on agmatinase was greater than on ADC, particularly by TGF-β, so that the ratio of ADC/agmatinase was increased by 46–102% over baseline, a shift favouring agmatine accumulation.

The effects of cytokines on LPS-mediated effects differed with respect to specific enzymes. In general, the cytokines tended to have little effect on the suppression of ADC activity by LPS. With the exception of TGF-β, cytokines reduced the elevation of agmatinase activity and facilitated the percentile increase of LPS-induced iNOS activity. In agreement with previous reports [16,19–21], IL-4 and IL-10 attenuated the induction of iNOS by LPS in absolute amounts of nitrate generated. However, while LPS reduced the ADC/agmatinase ratio in the absence of cytokines, the cytokines tended to neutralize this effect thereby promoting the net accumulation of agmatine.

Table 2 Effects of cytokines on the activity of iNOS, ADC and agmatinase in macrophages

Macrophages (RAW 264.7) were incubated in the presence of IL-4 (5 ng/ml), IL-10 (5 ng/ml) or TGF-β (2 ng/ml) for 20 h with or without stimulation by LPS (100 ng/ml). Results are expressed as means $\pm$ S.E.M. for five experiments. $^{\#}$ $P < 0.05$ compared with corresponding control (no LPS) group; * $P < 0.05$ compared with corresponding 'None' with (no treatment) group.

	[Nitrite] (nmol/mg of protein)		Activity (nmol/h per mg of protein)			
			ADC		Agmatinase	
Treatment	Control	LPS	Control	LPS	Control	LPS
None	1.91 ± 0.72	$10.9 \pm 0.62^{\#}$	25.1 ± 1.5	$13.4 \pm 2.7^{\#}$	7.4 ± 0.97	$13.3 \pm 0.4^{\#}$
IL-4	$0.26 \pm 0.13^{*}$	$6.77 \pm 0.37^{*}$	22.7 ± 4.6	15.3 ± 2.4	$4.3 \pm 0.92^{*}$	$4.14 \pm 0.62^{*}$
IL-10	$0.57 \pm 0.28^{*}$	$6.79 \pm 0.21^{*}$	$15.1 \pm 1.6^{*}$	11.4 ± 3.3	$2.81 \pm 0.4^{*}$	$2.77 \pm 0.87^{*}$
TGF-β	$0.71 \pm 0.25^{*}$	$10.8 \pm 0.83^{*}$	$13.4 \pm 3.8^{*}$	$6.09 \pm 2.6^{*}$	$1.88 \pm 1.0^{*}$	$4.49 \pm 1.37^{*}$

DISCUSSION

The present study sought to determine whether murine macrophage cells (RAW 264.7) express ADC and agmatinase, enzymes which synthesize agmatine from arginine and degrade it to putrescine respectively. If so, we sought to determine whether the enzymes are regulated in response to stimuli that modulate the expression of iNOS specifically LPS, a potent activator of macrophages [22] as well as IL-4, IL-10 and TGF-β, cytokines which inhibit macrophage activation [23]. Agmatine and its metabolic enzymes, ADC and agmatinase, prevalent in many lower lifeforms have only recently been discovered in mammals, and the physiological functions of agmatine are still being unravelled. As agmatine is a precursor of putrescine and hence of polyamines, some of its actions may relate to polyamine functions, e.g. induction of cell proliferation, differentiation and tissue regeneration. However there is also evidence the agmatine may act independently as a potential neurotransmitter/neuromodulator [10], as a secretogogue [1,8,9] and as an endogenous inhibitor of all isoforms of NOS [11].

We have discovered that both ADC and agmatinase are constitutively expressed in macrophages, with activities comparable with those reported in brain or kidney [1,4,5]. LPS dose-dependently and reversibly modulated the basal and evoked activity of both enzymes, as well as initiating induction of iNOS, indicating thereby that the enzymes are regulated. The action of the agent, moreover, was to promote changes in ADC and agmatinase by approximately halving the activity of the biosynthetic enzyme while nearly doubling the activity of the latter. This reciprocal pattern would have several effects: it would reduce the intracellular concentration of agmatine and thereby reduce the biosynthesis of putrescine by the agmatine-dependent pathway, reduce the concentration of the competitive endogenous inhibitor of iNOS, and as such promote availability of arginine into the NOS pathway.

Macrophages can be stimulated to express iNOS, which metabolizes L-arginine to citrulline and NO, which is highly reactive and cytotoxic [22]. On the other hand they also express arginase activity, forming urea and ornithine. Ornithine is decarboxylated by ornithine decarboxylase to putrescine and other polyamines and, until recently, this was believed to be the only pathway for polyamine biosynthesis in mammals. Arginine switches one pathway to another in order to sustain the double role of macrophages as destroyers of micro-organisms and tumour cells or as promoters of tissue repair. That the regulation of these metabolic routes is highly co-ordinated is supported by the facts that N^{G}-hydroxy-L-arginine, an intermediate of the reaction catalysed by iNOS, is a strong inhibitor of arginase [23,24] and that up-regulation of iNOS correlates with a decrease in arginase activity and vice verse [23,25,26].

The agmatine metabolic pathway may represent a third alternative pathway processing arginine in macrophages. When macrophages are activated by LPS, arginine transport [27–29] and the synthesis of arginine from citrulline by arginosuccinate synthase are stimulated [30], thus resulting in an increase in intracellular arginine. Once inside the cell, L-arginine is primarily utilized by iNOS for, as we have shown, like arginase [27], LPS reduces ADC activity. Agmatinase activity is enhanced, which will reduce accumulation of agmatine, an inhibitor of iNOS [11], by promoting its hydrolysis. Thus the presence of LPS in the medium would shunt arginine to the formation of NO by inhibiting other pathways for arginine degradation, by stimulating the hydrolysis of iNOS inhibitors and by increasing arginine synthesis and transport. While it has been established that the LPS-induced increases of iNOS and arginosuccinate synthase [30] involve gene transcription, the regulatory sites for the L-arginine transporter, arginase, ADC and agmatinase remain to be determined. However, that they may share a common regulatory mechanism is suggested by the correlation between induction of iNOS and agmatinase and ADC.

IL-10 [20,21], TGF-β [13,17] and IL-4 [15] can suppress the inflammatory activation of macrophages, thereby inhibiting NO production. Our results confirm the effects on iNOS of IL-10 and IL-4, although we did not detect changes with TGF-β, a finding supporting a study [31] which demonstrated that iNOS in resident peritoneal macrophages was not affected by exposure to TGF-β. As we demonstrate, IL-10 and TGF-β decreased ADC and agmatinase activities in unstimulated cells, whereas IL-4, while not changing ADC activity, reversed the effect of LPS on agmatinase. The fact that LPS-mediated changes in ADC and agmatinase activities were observed only after 20 h of incubation suggests that the effects of cytokines may result from a shift in the time course of LPS response. As we tested cytokines at only one time-point (20 h), further stories are required to verify whether cytokines delay the LPS-mediated effects on ADC and agmatinase. In general, the inhibitory cytokines had a suppressive effect on arginine metabolism. The mechanisms of action are still undetermined, but regulation at multiple cellular sites should be expected on the basis of current knowledge of the effect of inhibitory cytokines on iNOS expression. Thus TGF-β reduces the expression of iNOS protein by decreasing stability and translation of iNOS mRNA and by stimulating degradation of the enzyme [18], while IL-4 destabilizes it by interfering with transcription [19].

Unlike ADC and iNOS, the other L-arginine-dependent enzyme, arginase, is upregulated by TGF-β, IL-4 and IL-10 [25,26].

Arginase, therefore, is the most probable source of the increase in putrescine release induced by TGF-β [25,32], suggesting that this enzyme might be the preferred pathway through which L-arginine is metabolized when macrophages are not producing NO.

In summary, the present study shows that ADC and agmatinase are expressed in macrophages, that they are modulated in concert with other enzymes of arginine metabolism, possibly to accommodate to the different roles played by macrophages. Thus agmatine and the enzymes of its metabolism could play a role in inflammation by regulating iNOS activity and NO production.

We thank Ms. Liubov Lyandvert for cell culture preparations. M.S. was a recipient of a fellowship from the North Atlantic Treaty Organization (NATO).

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Received 26 August 1997/7 November 1997; accepted 29 December 1997