Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of arginine

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Agmatine, decarboxylated arginine, is a metabolic product of mammalian cells. Considering the close structural similarity between L-arginine and agmatine, we investigated the interaction of agmatine and nitric oxide synthases (NOSs), which use L-arginine to generate nitric oxide (NO) and citrulline. Brain, macrophages and endothelial cells were respectively used as sources for NOS isoforms I, II and III. Enzyme activity was measured by the production of nitrites or L-citrulline. Agmatine was a competitive NOS inhibitor but not an NO precursor. K_i values were approx. 660 μM (NOS I), 220 μM (NOS II) and 7.5 mM (NOS III). Structurally related polyamines did not inhibit NOS activity. Agmatine, therefore, may be an endogenous regulator of NO production in mammals.

INTRODUCTION

Agmatine, 4-(aminobutyl)guanidine, is produced by decarboxylation of L-arginine by the enzyme arginine decarboxylase. Although previously believed to be restricted to bacteria, plants and invertebrates [1], agmatine and arginine decarboxylase have been recently discovered in mammalian organs [2–4]. Henceforth, evidence has accumulated that agmatine is biologically active in mammals. It stimulates the release of catecholamines from adrenal chromaffin cells [2], increases arterial blood pressure when injected intracisternally in rats [5], stimulates the release of insulin [6], increases the release of luteinizing hormone-releasing hormone from hypothalamus [7], enhances morphine analgesia [8], and prevents tolerance to morphine [8]. Although it is known that agmatine is a ligand of α_2-adrenergic and imidazoline receptors [2,9] and a precursor of putrescine and other polyamines [10], the cellular mechanism by which agmatine exerts its biological actions is for the most part not established.

Here we examined the effects of agmatine on the production of nitric oxide (NO). NO, a regulator of haemostasis, immune defence and neurotransmission, is produced from L-arginine by a group of NO synthases (NOSs) that include three isoforms referred to here as NOS I, II and III [11,12]. Although all NOS isoforms have common substrate and cofactor requirements, they significantly differ in their cellular localization, mechanisms of regulation and functions [11,12]. NOSs generate NO by sequential oxidation of the guanidinium group in L-arginine [13]. Since agmatine is an L-arginine analogue with an intact guanidinium group we sought to determine (a) whether agmatine competes with the amino acid for NOS; (b) if so, whether it acts at all three isoforms; and (c) whether it is a substrate for NOS to produce NO. We report here that agmatine is a competitive inhibitor of all NOS isoenzymes but not an NO precursor.

MATERIALS AND METHODS

NOS I was analysed in homogenates of whole brain freshly obtained from male Sprague–Dawley rats killed by decapitation. NOS II was obtained from the macrophage RAW 264.7 cell line (a gift from Dr. Carl Nathan, Cornell University Medical College, New York, U.S.A.) maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum and 1 mM glutamine. The enzyme was induced by incubating cells overnight with 1 μg/ml lipopolysaccharide (LPS) [14]. NOS III was measured in bovine pulmonary artery endothelium (CPAE, from the American Tissue and Cell Collection) maintained in DMEM supplemented with 20 % (v/v) fetal calf serum, and used between passages 15 and 20. Cells were washed in ice-cold PBS, pH 7.6, scraped out of the flasks, and collected by a 5-min centrifugation at 1000 g at 4 °C.

All tissues were homogenized in 5–10 vol. of 50 mM Tris/HCl, pH 7.8, containing 1 mM DL-dithiothreitol. In order to remove endogenous amines and calcium, homogenates were mixed with the anionic exchanger Dowex-50W (Sigma) at pH 7.0, centrifuged in a microfuge at 12 000 g for 5 min and the supernatant used for enzyme activity measurements [14].

NOS activity was measured by production of [^{14}C]L-citrulline from [^{14}C]L-arginine [14]. Samples (50 μl) of tissue homogenate were incubated for 10 min at 37 °C in the presence of 50 mM Tris/HCl, pH 7.8, containing 0.5 mM NADPH, 5 μM FAD, 5 μM tetrahydrobiopterin (BH$_4$), 50 nCi (1.7 μM) of [^{14}C]L-arginine (305 mCi/mmol; Amersham), 10 μM CaCl$_2$ and 10 μg/ml calmodulin, in a total volume of 100 μl. The reactions were halted by addition of 0.4 ml of ice-cold 20 mM Hepes, pH 5.5, mixed with 1 ml of Dowex-50W equilibrated with Hepes and briefly centrifuged. [^{14}C]L-Citrulline was recovered in the supernatant and its concentration determined by liquid scintillation counting. Blanks were samples assayed in 200 μM L-N^G-monomethyl-L-arginine (L-NMMA).

NOS was also assessed in macrophage homogenates by measuring the accumulation of nitrites. Aliquots (50 μl) of homogenate from LPS-treated RAW cells were incubated with 50 μl of reaction mixture containing ions and cofactors as described above, and either unlabelled arginine or agmatine. After 5 h at 37 °C, samples were centrifuged briefly and the supernatant mixed with 80 μl of Griess reagent [15], incubated at room temperature for 15 min, and the absorbance at 570 nm determined in a microplate reader.

RESULTS AND DISCUSSION

As expected, NOS activity, measured as production of L-citrulline (pmol/mg of protein per 10 min) was constitutively present in whole rat brain (520 ± 25) and bovine cultured pulmonary endothelial cells (168 ± 15), and was induced by LPS in macrophages (414 ± 40). Activity was inhibited by the specific NOS inhibitor L-NMMA to 32.3 ± 5.4 (NOS I), 50.2 ± 16.3 (NOS II) and 5.3 ± 0.5 (NOS III). EGTA reduced NOS activity in brain (40.3 ± 8.3) and endothelial cells (12.5 ± 1.3), but not in macrophages (375 ± 43), thus confirming the calcium-dependent nature of both NOS I and NOS III but not NOS II [11,12]. (Values presented above are means $\pm$ S.E.M, $n = 3$).

Agmatine inhibited in a dose-dependent manner the activity of the three NOSs (Figure 1A). The inhibition of NOS I and NOS II was complete at the highest doses, while at maximum doses NOS III was only reduced by 60 %. While agmatine might, because of its structural similarity to L-arginine, compete with this substrate at the NOS binding site, agmatine is also a highly basic molecule which might interact with acidic constituents elsewhere in the NOS protein. To discriminate between these two possibilities, we examined the kinetics of the interaction between
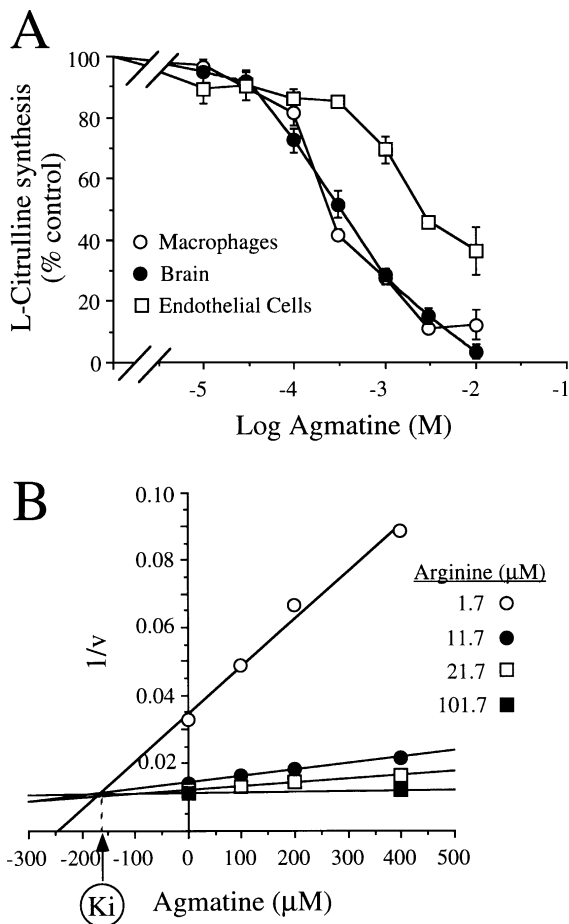
Table 1   NO production by NOS II using L-arginine and agmatine as substrates

Macrophage homogenates were used as the enzyme source. NO production was estimated as the accumulation of nitrites (nmol/mg of protein per 5 h). Values are means $\pm$ S.E.M., $n = 3$. Statistical significance: *$P < 0.05$ compared with control (Student's t test for paired data).

Concentration (M)	Accumulation of nitrites (nmol/mg of protein per 5 h)	
	Arginine	Agmatine
0	4.0 ± 0.9	3.8 ± 0.9
10^{-5}	5.4 ± 0.1	3.9 ± 1.0
10^{-4}	$15.7 \pm 4.2^*$	3.6 ± 0.6
10^{-3}	$21.1 \pm 5.2^*$	3.4 ± 0.6
10^{-2}	$18.4 \pm 3.0^*$	3.0 ± 0.6

Figure 1   Inhibition of NOS activity by agmatine

(**A**) Dose-dependent inhibition of NOS I (brain), NOS II (macrophages), and NOS III (endothelial cells) by agmatine in the presence of 1.7 μM arginine. NOS activity was measured as the production of [^{14}C]L-citrulline from [^{14}C]L-arginine in tissue homogenates. Values are the means $\pm$ S.E.M. of 3–6 determinations. Absolute values of enzyme activity are indicated in the text. (**B**) Representative Dixon plot for NOS II in the presence of 1.7–101.1 μM arginine.

agmatine and NOS, and the effect on NOS activity of polyamines sharing comparable electrostatic properties.

Kinetic analysis using either Lineweaver–Burk or Dixon plots revealed that inhibition was competitive (Figure 1B). NOS II showed the lowest K_i (218 ± 23 μM, $n = 4$; mean $\pm$ S.E.M), followed by NOS I (664 ± 107 μM, $n = 4$) and NOS III (7.5 ± 0.4 mM, $n = 3$). In addition, polyamines such as putrescine, spermine, spermidine and ornithine failed to inhibit any of the NOSs, even at concentrations up to 10^{-3} M ($n = 3$). Thus, agmatine appears to compete with L-arginine at the catalytic binding site.

NO originates from the guanidinium moiety of L-arginine [13]. Since agmatine also has a guanidinium group, it was conceivable that NOS could metabolize agmatine into NO and decarboxylated L-citrulline. To test this possibility we measured the production of nitrites, metabolic end-products of NO [11,15], in macrophage cell homogenates incubated with either agmatine or arginine. Agmatine, unlike arginine, did not increase nitrite production over basal levels (Table 1). This finding indicates that agmatine is not a precursor for NO, and illustrates the importance, still unexplored, of the carboxyl moiety of L-arginine in the reaction catalysed by NOSs.

We conclude that agmatine competitively inhibits NOSs, most potently the inducible isoform. Multiple NOS inhibitors have been developed in the last few years to unravel the physiology of NO, as well as to specifically block pathological production of this molecule [16]. Most of these drugs are L-arginine analogues with an affinity for NOSs similar to or lower than L-arginine, whose apparent K_m values range between 1 and 30 μM [16]. Although agmatine is a weaker NOS inhibitor, its relevance lies in the fact that this amine is naturally occurring in mammalian systems. Initially described in brain [2], agmatine has been detected in numerous organs, and is particularly abundant in stomach, small intestine and aorta [3,17]. Considering that agmatine binds NOSs with significantly lower affinity than L-arginine, effective competition is only conceivable if agmatine is more concentrated than L-arginine. A comparison of the distribution of the two amines in different organs reveals that L-arginine may be 4–50 times more abundant, in nmol/g wet tissue, than agmatine [3,18]. Although this observation might imply that agmatine is a weak regulator of NOS activity, the values do not necessarily reflect either the amine concentrations at NOS catalytic sites, or their fluctuations in different physiological and pathological situations. Recent evidence indeed suggests that conditions may exist in which agmatine may become

a more effective competitor of arginine. Thus, in cultured astrocytes interferon-γ increases by 50 % the activity of arginine decarboxylase [19], and agmatine is 20-fold higher in rat aorta after ischaemic injury [17]. Moreover, methyl-arginines, which are also endogenous NOS inhibitors, [18,20] are normally over 500 times less abundant than L-arginine [18], but in the course of renal failure their concentrations rise enough to block NOS II and NOS III, thereby causing immune dysfunction and hypertension [20].

Agmatine can be available to NOS *in vivo* in two possible ways. First, agmatine could act in the same cell where it is produced. Indeed, arginine decarboxylase has been detected in cells that express constitutive or inducible forms of NOS [19,21]. Secondly, agmatine could be sequestered in cells that lack arginine decarboxylase activity. Since agmatine is present in appreciable amounts in blood and presumably other extracellular fluids [3], it would hence be potentially accessible to all cells expressing any isoenzyme of NOS. However, no transport systems for agmatine have yet been reported in mammalian cells. An alternative mode of entry could be through activated cation channels, as has been shown in invertebrate and mammalian sympathetic neurons [22,23], although comparable evidence is not available in central neurons, macrophages or endothelial cells.

Whatever the source, agmatine has been detected in neurons [24] and endothelial cells [20], the characteristic hosts for NOS I and NOS III, as well as in macrophages (S. Regunathan, unpublished work) or astrocytes [19], which express NOS II *in vivo* and *in vitro* when exposed to inflammatoty cytokines and bacterial endotoxins [11,12,14]. The cellular co-localization of agmatine and NOSs supports the view that this amine may be an endogenous regulator of the production of NO, and raises the possibility that some of the biological effects mediated by agmatine [2,5–8] might be due to interaction with the ubiquitous NO system. It also suggests that mechanisms regulating either arginine decarboxylase or the enzyme that degrades agmatine (agmatinase), which we have recently found in mammals [10],

may also be of relevance in the regulation of NO production in health and disease.

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