Communication

Agmatine Suppresses Proliferation by Frameshift Induction of Antizyme and Attenuation of Cellular Polyamine Levels*

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Polyamines are required for entry and progression of the cell cycle. As such, augmentation of polyamine levels is essential for cellular transformation. Polyamines are autoregulated through induction of antizyme, which represses both the rate-limiting polyamine biosynthetic enzyme ornithine decarboxylase and cellular polyamine transport. In the present study we demonstrate that agmatine, a metabolite of arginine via arginine decarboxylase (an arginine pathway distinct from that of the classical polyamines), also serves the dual regulatory functions of suppressing polyamine biosynthesis and cellular polyamine uptake through induction of antizyme. The capacity of agmatine to induce antizyme is demonstrated by: (a) an agmatine-dependent translational frameshift of antizyme mRNA to produce a full-length protein and (b) suppression of agmatine-dependent inhibitory activity by either anti-antizyme IgG or antizyme inhibitor. Furthermore, agmatine administration depletes intracellular polyamine levels to suppress cellular proliferation in a transformed cell line. This suppression is reversible with polyamine supplementation. We propose a novel regulatory pathway in which agmatine acts as an antiproliferative molecule and potential tumor suppressor by restricting the cellular polyamine supply required to support growth.

Polyamines (putrescine, spermidine, and spermine) are required for DNA replication, proliferation, and cell homeostasis (1–3). Ornithine decarboxylase (ODC)1 is the first rate-limiting enzyme of polyamine biosynthesis and one of the most highly regulated eukaryotic enzymes. Cellular polyamine transporters are stimulated by many of the same factors that induce ODC activity, and similarly, enhanced cellular polyamine uptake occurs both in normal but rapidly proliferating cells (4) and in tumor cell lines (5–8). Cells in vivo can acquire polyamines released into the circulation by other cells, dietary sources, and gut flora. Polyamines have been demonstrated to play an important role in the transformation process. Conversely, polyamine depletion results in growth arrest (9, 10).

Intracellular polyamine concentrations are autoregulated by the induction of the protein antizyme (11). Antizyme is the only known endogenous protein that binds to ODC, inhibiting activity and accelerating its degradation (12). In addition to inhibiting polyamine biosynthesis, antizyme has recently been shown to concurrently suppress polyamine transporter(s) (13, 14). Pharmacological inhibition of ODC activity, however, has been shown to result in compensatory cellular polyamine uptake (6). Beneficial therapeutic intervention must therefore address both polyamine transport as well as biosynthesis (for review see Ref. 15).

The metabolism of arginine to agmatine by ADC has only recently been demonstrated in mammals (16). As agmatine and polyamines are structurally analogous polycationic molecules derived from distinct arginine-dependent pathways (6), we speculated that the ADC metabolite agmatine may play a role in regulating intracellular polyamines. Herein we report concurrent suppression of both polyamine biosynthesis and transport by agmatine. This occurs primarily via induction of antizyme and results in attenuated proliferation in a transformed cell line.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma unless otherwise stated. We were unable to detect impurities by HPLC analysis in agmatine purchased from Aldrich. Sigma agmatine was found to have 3–8% impurities.

Cells and Cell Cultures—All cell lines were from American Type Culture Collection except MCT (mouse kidney proximal tubule) (17), Ras-3T3 (Ras transformed NIH-3T3 fibroblast) (18), mMC (mouse glomerular mesangial) (19), JS-1 (human Schwann tumor) (20), and ENDO (rat glomerular endothelial) (21). Cells were maintained in culture in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum (HyClone Laboratories). Experiments were performed when incubation of cell cultures in 10-cm dishes reached 80–95% confluence, unless otherwise noted.

ODC Activity—Cells were washed and resuspended in ODC reaction buffer (10 mM Tris, pH 7.4, 2.5 mM dithiothreitol, 0.3 mM pyridoxal-5-phosphate, 0.1 mM EDTA) and homogenized by polytron. The homogenate was centrifuged at 30,000 × g for 40 min, and the supernatant was assayed for ODC activity as described (22).

Transport Studies—Cells were cultured (6-well plates) in the absence or presence of 1 mM agmatine for the indicated times (see Fig. 2). Cells were then washed three times with phosphate-buffered saline prior to a 15-min incubation with 0.5 μCi ml⁻¹ 12.5 nM [3H]putrescine (NEN Life Science Products) in Dulbecco’s modified Eagle’s medium. Five cold washes of phosphate-buffered saline terminated the incorporation. Cells were lysed with 1 N NaOH and counted in a β scintillation counter.

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1 The abbreviations used are: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; HPLC, high pressure liquid chromatography.
Antizyme Frameshifting—Fusion antizyme mRNAs were used in a reticulocyte lysate assay as described previously (11). Frameshift efficiencies were calculated from the radioactivities of frameshift and termination bands with a BAS 2000 image analyzer.

Antizyme Antibody and Antizyme Inhibitor Assays—MCT cells were incubated with 5 mM agmatine and 1 mM aminoguanidine for 24 h. Aminoguanidine was used to inhibit serum diamine oxidase, an enzyme that metabolizes agmatine. Preparation of ODC, anti-antizyme antibody, and antizyme inhibitor methodology were as described previously (23).

Proliferation Assays—15,000 (see Fig. 4A) or 5000 (see Fig. 4D) MCT cells/well on a 96-well plate were incubated in the absence or presence of 1 mM agmatine and 0.05 mM putrescine for 24 or 48 h as indicated in the figures. [3H]Thymidine (1 μCi/well) was added 16 h prior to harvesting in an automated multiwell harvester and β scintillation counting. Cell counting (see Fig. 4B) was performed by plating 50,000 MCT cells/well of a 24-well plate in the absence or presence of agmatine (concentrations indicated in the figure legend). Cells were collected and counted in a hemocytometer using trypan blue exclusion to identify viable cells.

Conversion of Agmatine to Polyamines—MCT cells (10-cm plate) were incubated with [3H]agmatine (10 μCi/ml Dulbecco’s modified Eagle’s medium ± 5% fetal calf serum; American Radiolabeled Chemicals Inc.) for a 2-h incubation period. Cells were washed, collected, scraped, lysed, and prepared for HPLC as described below.

Determination of Intracellular Polyamine Levels—Cellular extracts were deproteinized prior to derivatization (AccQ-Fluor kit, Waters Corp.) and HPLC elution on a C18 reverse phase column (ODS Hypersil 3 μm). The HPLC flow rate was 0.5 ml/min, and the elution gradient was as follows: 5% A 0–3 min, 7% A 3–10 min, 7–30% A 10–30 min, and 30–50% A 30–35 min. Buffer A was supplied in the AccQ-Fluor kit, and buffer B was HPLC grade acetonitrile. Polyamines were detected by fluorescence as per AccQ-Fluor kit instructions (see Fig. 4C) or radioactivity assessed for [3H]agmatine conversion (not shown).

RESULTS

Agmatine Suppresses ODC Activity—Because the kidney is a principal site of ADC activity, we performed these experiments in a mouse kidney proximal tubule cell line, MCT (17). This transformed cell line demonstrates elevated constitutive ODC activity and diminished ADC activity compared with freshly isolated proximal tubule cells. These aspects make the MCT cell line an appropriate model system for observing the effects of agmatine supplementation on ODC activity and intracellular polyamine levels. Exogenous agmatine administration to MCT cells for 18 h resulted in a concentration-dependent suppression of ODC activity (Fig. 1A). Agmatine was not toxic at these or higher concentrations as determined by trypan blue exclusion. Agmatine purchased from either Aldrich or Sigma yielded comparable results. The effects on ODC activity were evident following a 30-min lag period (Fig. 1B). Because agmatine is distributed by the plasma and can be concentrated in tissues (34), we examined whether its effects are cell type-specific. Fig. 1C demonstrates the efficacy of exogenous agmatine administration for 18 h in suppressing ODC activity in multiple immortalized or transformed cell lines.

Agmatine Suppresses Polyamine Transport—The rapid polyamine transport observed in MCT cells as determined by [3H]putrescine uptake is indicative of active polyamine transporters (Fig. 2, 0 h of agmatine preincubation). To determine whether agmatine affects polyamine transporters, cells were preincubated in the presence of agmatine and then shifted to agmatine-free medium containing [3H]putrescine. Extracellular agmatine was removed during the labeled uptake period of this experiment to demonstrate suppression of transporters and avoid potential competitive effects of agmatine with [3H]putrescine (Fig. 2).

Induction of Antizyme by Agmatine—Polyamines autoregulate their intracellular concentrations through the induction of antizyme, which suppresses both polyamine biosynthesis and transport. It has been demonstrated that polyamine induction of antizyme is not primarily a transcriptional event but rather occurs by a distinct mechanism of programmed ribosomal frameshifting (11). We utilized a rabbit reticulocyte lysate assay to examine whether agmatine can directly induce a +1 translational frameshift of antizyme mRNA in vitro. These results are depicted in Fig. 3A. The frameshift efficiency, and therefore the effectiveness of producing full-length, functional antizyme by 4 mM agmatine, was comparable with that of 4 mM putrescine and 0.5 mM spermidine. These concentrations of the canonical polyamines represent at or near maximal effective concentrations for this assay as previously established (11). We have found that the effect of agmatine on frameshifting efficiencies was dose-dependent (not shown). We next examined whether antizyme induction is required for the effect of agmatine on ODC. Purified ODC enzymatic activity was markedly inhibited in the presence of agmatine-treated (MCT) cell extracts. The ODC inhibitory activity of agmatine-treated cell extracts was precipitated with the anti-antizyme antibody but not with control IgG (Fig. 3B). Furthermore, addition of highly purified antizyme inhibitor (24) also attenuated the inhibitory effect of the cell extracts (Fig. 3B). Together, these results demonstrate that agmatine is capable of inducing antizyme.

Agmatine Administration Suppresses Proliferation—Intracellular polyamines are maintained in reserve, allowing pas-
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**DISCUSSION**

Polyamines are required components for both protein and nucleic acid synthesis, and polyamine biosynthesis is regulated throughout the course of the cell cycle (26, 27). Antizyme, through its ability to suppress both the polyamine biosynthetic enzyme ODC and polyamine transporters, is an effective endogenous mechanism for limiting intracellular polyamine levels. This unique, two-pronged negative feedback system of antizyme activity limits further cell division and proliferation in agmatine-treated cells, as verified by cell counting, was maintained throughout a 10-day experiment (Fig. 4B).

To further define the effects of agmatine on intracellular polyamine levels, we analyzed intracellular polyamine levels by HPLC. We first determined that the induction of antizyme was not due to a conversion of agmatine to polyamines. MCT cells were incubated for 2 h in the presence of [3H]agmatine, which corresponds to the time of near maximal inhibition of ODC activity by agmatine. We observed no conversion of [3H]agmatine to any of the polyamines at this time by HPLC determination (not shown). We then determined the effects of agmatine on intracellular polyamine levels after 24–48 h of administration, corresponding to the time points of [3H]thymidine experiment. In the absence of agmatine, MCT cells demonstrated a significant spermidine peak. Intracellular spermidine is markedly reduced in cells exposed to agmatine for 24 (41%) or 48 h (82%) (Fig. 4C). Putrescine levels are also significantly reduced, resulting in a substantial reduction in total intracellular polyamine levels.

To determine the specificity of the inhibitory effects of agmatine, we administered a low dose of putrescine concurrently with agmatine and examined [3H]thymidine incorporation at 48 h. Putrescine effectively prevented agmatine inhibition of [3H]thymidine incorporation at 48 h, thereby demonstrating specificity of the inhibition and supporting the conclusion that agmatine effects are the result of polyamine limitation (Fig. 4D).

**Fig. 2. Polyamine transport is inhibited in the presence of agmatine.** Preincubation of MCT cells with 1 mM agmatine for the indicated times was followed by phosphate-buffered saline washes prior to a 15-min 10 μM [3H]putrescine incubation.

**Fig. 3. Induction of antizyme by agmatine.** A, *in vitro* stimulation of translational frameshifting of a fusion antizyme mRNA by agmatine. The T7 transcripts from the wild-type fusion antizyme cDNA (C3NE) and its +1 in-frame control (ΔT205) were incubated in the presence of [35S]methionine alone (lane 1) or with the addition of 4 mM agmatine (lane 2), 4 mM putrescine (lane 3), or 0.5 mM spermidine (lane 4). FS indicates the full-length +1 frameshifted antizyme band, and TERM represents the terminated, nonactive antizyme product. B, antizyme activity in the extract of agmatine-treated MCT cells. The bars represent purified rat liver ODC in the presence of nontreated MCT cellular extract (column 1, black) or with the addition of agmatine-treated MCT cellular extract (Agm) (columns 2–5). Column 3, diagonally striped, agmatine-treated cellular extract plus anti-antizyme IgG; column 4, vertically striped, control IgG; column 5, cross-hatched, antizyme inhibitor.

Recent studies have suggested a correlation of agmatine binding to imidazoline (I₂) receptors with the selective inhibition of proliferation of cultured primary vascular smooth muscle cells (28). In our studies, inhibition of ODC activity was demonstrated in membrane-free preparations, which could not be attributed to a receptor-dependent mechanism. Agmatine exerted its maximum effects on proliferation at 1 mM (Fig. 4B). The capacity of agmatine to frameshift antizyme mRNA to produce a full-length protein was also observed at 1 μM. Although maximum frameshift efficiencies were at 4 mM (Fig. 3A), 1 μM may be sufficient to block ODC and polyamine transport causing maximal growth inhibition. Differences between the effect of agmatine in the frameshift and proliferation assays may also reflect differing concentrations of agmatine in the medium and in the cells. We are currently investigating how agmatine is transported and accumulated within the cell. Although we cannot entirely exclude potential nonantizyme-mediated effects by agmatine, antizyme is required for suppression of ODC activity (Fig. 3B). Furthermore, effects on proliferation were shown to be reversed if putrescine was administered concurrently with agmatine (Fig. 4D), thus supporting the premise of cellular polyamine limitation as the inhibitory mechanism. Although this latter result may initially appear inconsistent with the findings of concur-
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Fig. 4. Inhibition of DNA synthesis by agmatine in a transformed cell line. A, MCT cells in the absence (control) or presence of 1 mM agmatine incubated for 24 or 48 h (white bars). B, cell counting of MCT cells in the absence (control, †) or presence of 1 (■), 100 (○), or 1000 μM (△) agmatine for the days indicated. C, three representative HPLC chromatograms are overlaid to highlight changes in polyamine levels in cultured MCT cells following incubation in the absence (0 h) or presence of 1 mM agmatine for 24 or 48 h. D, [3H]thymidine incorporation in MCT cells incubated for 48 h in untreated (control, black bars), 1 mM agmatine-treated (white bar), or 1 mM agmatine plus 50 μM putrescine-treated (stippled bar) cells.

Fig. 5. Schematic diagram of the proposed pathway for agmatine induction of antizyme, with consequent suppression of polyamine biosynthesis (ODC) and transport. The oval in the cell membrane represents polyamine transporter. The bars represent negative regulation by antizyme.

current agmatine inhibition of polyamine transport, it should be noted that inhibition of cellular uptake was neither complete nor immediate, requiring at least 2 h for maximum effect (Fig. 2), allowing cellular putrescine to accumulate in these cells over this period. Our results also demonstrate that agmatine, at doses causing cellular polyamine depletion, does not act as a precursor of the canonical polyamines in terms of supporting cell proliferation (Fig. 4). The findings of this study corroborate earlier studies reaffirming the necessity of polyamines in maintaining a transformed state.

When ODC activity is induced in tissues such as liver and kidney following a hyperplastic or hypertrophic stimulus, there is a concurrent reduction in ADC activity.2 We observed similar reciprocal changes in the transformed proximal tubule cell line, MCT, compared with freshly isolated proximal tubule cells.3 Such correlations suggest a physiologic role for ADC metabolites in the control of cellular proliferation and as potential tumor suppressors. ADC activity could also serve to divert arginine from arginase, thereby limiting substrate for ODC and effectively compounding the effects of agmatine. Targeted gene disruption of ADC would be an important step in further understanding the functional importance of this pathway.

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