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Molecular Basis for the Antiproliferative Effect of Agmatine in Tumor Cells of Colonic, Hepatic, and Neuronal Origin

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ABSTRACT

The aim of the present study was to challenge potential mechanisms of action underlying the inhibition of tumor cell proliferation by agmatine. Agmatine inhibited proliferation of the human hepatoma cells HepG2, the human adenocarcinoma cells HT29, the rat hepatoma cells McRH7777, and the rat pheochromocytoma cells PC-12. Inhibition of proliferation of HepG2 cells was associated with an abolition of expression of ornithine decarboxylase (ODC) protein and a doubling of mRNA content encoding ODC. In HepG2 cells, silencing of ODC-antizyme-1, but not of antizyme inhibitor, by RNA interference resulted in an increase of agmatine's antiproliferative effect. Thus, the distinct decrease in intracellular polyamine content by agmatine was due to a reduced translation of the synthesizing protein ODC but was not essentially mediated by induction of ODC-antizyme or blockade of antizyme inhibitor. In interaction experiments 1 mM L-arginine, 1 mM D-arginine, 1 mM citrulline, 100 μM N^{ω}-nitro-L-arginine methyl ester, 1 and 10 μM sodium nitroprusside, and 1 μM N^{1}-guanyl-1,7-diaminoheptane failed to alter agmatine's antiproliferative effect. Hence, the antiproliferative effect of agmatine in HT29 and HepG2 cells is due to an interaction with neither the NO synthases, the hypusination of eIF5A, nor an agmatine-induced reduction in availability of intracellular L-arginine. L-Arginine and citrulline, but not D-arginine, inhibited tumor cell proliferation by themselves. Their inhibitory effect was abolished after silencing of arginine decarboxylase (ADC) expression by RNA interference indicating the conversion to agmatine by ADC. Finally, in the four cell lines under study, agmatine-induced inhibition of cell proliferation was paralleled by an increase in intracellular caspase-3 activity, indicating a promotion of apoptosis.

Agmatine, a cationic amine formed by decarboxylation of L-arginine by the mitochondrial enzyme arginine decarboxylase, initially attracted attention as an endogenous ligand at imidazoline receptors (Li et al., 1994). However, independent of binding to those receptors, agmatine induces a variety of physiological and pharmacological effects (Raasch et al., 2001). In particular, the antiproliferative effect of agmatine has aroused interest as a new alternative in the treatment of neoplasms. Agmatine administration to tumor cells in vitro results in a suppression of cell proliferation (Satriano et al., 1998; Choi and Cho, 1999; Vargiu et al., 1999; Babal et al., 2001; Dudkowska et al., 2003; Higashi et al., 2004; Kribben et al., 2004; Molderings et al., 2004; Mayeur et al., 2005). At the intracellular level, agmatine-induced decrease of cell proliferation was shown to be due to a decrease in the intracellular levels of the polyamines putrescine, spermidine, and spermine (Satriano et al., 1998; Choi and Cho, 1999; Vargiu et al., 1999; Babal et al., 2001; Dudkowska et al., 2003; Higashi et al., 2004; Mayeur et al., 2005), which are pivotal for cell growth (Igarashi and Kashiwagi, 2000). The intracellular concentration of polyamines is controlled at several stages, including their biosynthesis and their uptake. The former is mainly achieved by controlling the cellular ornithine decarboxylase (ODC) activity at the post-translational level through a family of at least four inhibitory proteins called ODC-antizymes (ODC-Az1 to ODC-Az4) (Mangold, 2005). ODC-Az1 disrupts enzymatically active ODC homodimers by forming ODC-antizyme heterodimers promoting the degradation of ODC by the 26S proteasome in a ubiquitin-independent manner. In addition, ODC-Az1 inhibits its polyamine uptake and stimulates polyamine excretion. Binding of ODC-Az2 does not result in the degradation of ODC but inhibits cellular polyamine uptake. The modes of

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ABBREVIATIONS: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; AZI, antizyme inhibitor; eIF5A, eukaryotic translation initiation factor 5A; GC7, N^{1}-guanyl-1,7-diaminoheptane; L-NAME, N^{ω}-nitro-L-arginine methyl ester; ODC-Az, ornithine decarboxylase antizyme-1; PCR, polymerase chain reaction; siRNA, small interfering RNA.

action of ODC-Az3, whose expression is limited to the testis, and of ODC-Az4 have not yet been analyzed in detail. The functions of all antizyme proteins are modulated by an inhibitory protein, called antizyme inhibitor (AZI) (Mangold and Leberer, 2005). AZI shares homology with ODC but lacks any enzymatic activity. Antizymes bind AZI with higher affinity than they bind to ODC, which in turn causes the release of ODC from antizyme inhibition. The negative regulation of ODC by agmatine has been shown to be associated in some but not all cell types, with the induction of the protein ODC-antizyme. Hence, the necessity of antizyme protein induction for agmatine-induced decay of ODC activity and inhibition of polyamine uptake still is an open question.

Regarding the proliferative effects of polyamines in mammalian cells, it is supposed that the stimulation of protein synthesis by polyamines may partially be dependent on the formation of active eIF5A (Jakus et al., 1993; Tome and Gerner, 1997) because eIF5A contains the amino acid hypusine, which is exclusively derived from spermidine (Park et al., 1997). eIF5A is a universally conserved protein that seems to be closely associated with cell proliferation in various mammalian cells (Park et al., 1997; Tome and Gerner, 1997). Although the precise role of eIF5A in protein synthesis or other cellular pathways remains to be clarified, the prevailing hypothesis suggests eIF5A to be a ribosome-associated, mRNA-specific translation factor that stimulates ribosome function. Hence, the antiproliferative effect of agmatine might be due, at least in part, to the distinct agmatine-induced decrease in the intracellular content of spermidine and consecutively of eIF5A.

Finally, it is conceivable that, in addition, the antiproliferative effect of agmatine may be due in part to interactions with the NO system (Satriano, 2004). The aim of the present study was to challenge these unproven hypotheses on agmatine's action at intracellular targets, thus providing an integrative explanation of the conflicting results obtained in different cell types.

Materials and Methods

Cell Culture. The rat hepatoma cell line McRH7777 was obtained from American Type Culture Collection (Manassas, VA); the human hepatoma cell line HepG2 and the human intestinal tumor cell line HT29 were generous gifts of the Department of Internal Medicine (University of Bonn, Bonn, Germany). Cells were grown in a humidified atmosphere of 5% CO_2 in plastic culture dishes (22 mm; Nalge Nunc International, Wiesbaden, Germany) using Dulbecco's modified Eagle's medium (HT29, McRH7777) and RPMI 1640 (HepG2) medium (Sigma, Munich, Germany) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (Biochrom, Berlin, Germany). The rat pheochromocytoma PC-12 cells were cultured as adherent cells in a humidified CO_2 incubator (at 37°C and in the presence of 9% CO_2) in plastic culture dishes (Nalge Nunc) coated with polyornithine 0.1 g/l (in 0.15 M boric acid and 67 mM NaOH, adjusted with HCl to pH 8.4) using RPMI medium supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 10% fetal calf serum, and 5% horse serum (Biochrom).

Proliferation Assay. The cells were incubated in the absence (control cells) or presence of the drugs under study for 72 h unless stated otherwise. At the end of the experiment the protein content of each well was determined by the method of Bradford (1976) as an estimate for the cell number. Protein content has been shown to be an adequate surrogate for cell proliferation (Molderings et al., 2004). Toxic effects of the compounds under study on the cells were visualized and ruled out be the trypan blue exclusion test.

Caspase-3 Assay. Caspase-3 activity was assessed by colorimetric assay according to the manufacturer's protocol (BioVision, Mountain View, CA).

Experiments with RNA Interference. The sequences of the small interfering RNAs (siRNAs) were chosen from the respective human reference sequences according to published design guidelines with dTdT 3′ overhangs. Sequences for the sense strand of the central 19-nucleotide double-stranded region were the following: human ODC-antizyme-1 (Genbank accession no. NM_004152), CCUUCAGCUUUUUGGGCUUU; human ODC-antizyme inhibitor (Genbank accession no. NM_015878), UUGCACGUAAUCACCCAAA; and human arginine decarboxylase (AY325129), GAAACCAUCCACGGAGCAG. All siRNAs target the open reading frame. The siRNAs targeting human antizyme-1, antizyme inhibitor, and ADC have been proven selective and effective by Newman et al. (2004), Choi and Cho (2005), and Molderings (2006), respectively. A sequence targeting antizyme-1 was chosen for the RNA interference because antizyme-1 is the antizyme that is involved in the feedback mechanism of the polyamines and probably of agmatine on cell growth (for a detailed discussion, see Introduction). siRNAs were synthesized by MWG Biotech (Ebersberg, Germany). The desalted and purified siRNA duplex was mixed with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) at a ratio of 200 picomoles of siRNA/2 μl of Lipofectamine 2000 according to the manufacturer's instructions. Each well containing cells that had been in culture for 3 days received either 2 μl of Lipofectamine alone (controls) or 2 μl of Lipofectamine and 200 picomoles of siRNA in a total volume of 500 μl of culture medium containing the respective serum supplement but no antibiotics. The efficacy of transfection was monitored by using the Block-iT Fluorescent Oligo Kit (Invitrogen) according to the manufacturer's protocol. The efficacy of the RNA interference was monitored by comparison of the expression of the respective mRNA in cells incubated only with the transfection reagent with those transfected with the respective siRNA by quantitative PCR. Transfection with the siRNAs resulted in a decrease of the corresponding mRNA by approximately 70% (geometric mean; range, 23.0–96.5%; coefficient of variation, 62%; $n = 5$ in each series).

Quantitative Polymerase Chain Reaction. RNA from HepG2 cells was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) with DNase treatment according to the manufacturer's instructions. Total RNA of each sample was reverse-transcribed according to the manufacturer's instructions (Superscript II, Invitrogen; random hexamer primers, MWG). For quantitative PCR, 35 μl of amplification mixture (QuantiTect SYBR Green Kit; QIAGEN) was used, containing 50 ng of reverse-transcribed RNA and 300 nM concentrations of the respective primers (Table 1) according to the manufacturer's instructions. Reactions (triplicates, 10 μl) were run on an Mx3000 real-time cycler (Stratagene, La Jolla, CA). The cycling conditions were the following: 15 s polymerase activation at

TABLE 1
Primers

ODC	
Forward	5′-GTGGGTGATTGGATGCTCTTTG-3′
Reverse	5′-AGGCCCTGACATCACATAGTAG-3′
ADC	
Forward	5′-CTGCCGCAACTACACGTAG-3′
Reverse	5′-GGACATGGCATAGGTGATGTG-3′
ODC-antizyme-1	
Forward	5′-CGAGGATTCTCAACGTCCAG-3′
Reverse	5′-GATCTCGATGTAGAGGCTGC-3′
Antizyme inhibitor	
Forward	5′-TGATGCAAACTACTCCGTTGGC-3′
Reverse	5′-GAGCCACTACATTCTGCCATTG-3′
β-actin	
Forward	5′-TCCATCATGAAGTGTGACGT-3′
Reverse	5′-GAGCAATGATCTTGATCTTCAT-3′

95°C and 45 cycles at 95°C for 15 s, at 58°C for 30 s, and at 72°C for 30 s. Each assay included a standard curve (5 points from 200 to 12.5 ng/35 μl) and no-template controls. The results were analyzed using the Stratagene software (version Mx3000 Pro). The relative mRNA expression (R) was calculated according to Pfaffl (2001) from the ratio "treated cells" over "control cells," $R = E^{\text{ct control-treated}}$ (target)/$E^{\text{ct control-treated}}$ (housekeeper) with the efficiency $E = 10^{-1/\text{slope}}$ measured with a standard curve in all experiments. The results for the housekeeping gene β-actin were determined by the same method (β-actin primers; Table 1). The housekeeping gene that is stably expressed in all samples was used as an internal standard to normalize mRNA expression, which compensates differences in sample concentrations and reverse-transcription efficiencies. However, in the present experiments, no significant difference was detected comparing normalization to β-actin and to total RNA amount, showing negligible variations in reverse transcription and PCR efficiencies. The identity of the PCR products was initially confirmed by agarose gel electrophoresis followed by dideoxy chain termination sequencing and then after each real-time reaction by melt-point analysis.

Determination of ODC by Western Blotting. HepG2 cells were grown in the absence or presence of 1 mM agmatine for 72 h. Cells were harvested and lysed in 1% SDS-polyacrylamide gel electrophoresis buffer, and 50 μg of protein were separated by SDS-Disk-polyacrylamide gel electrophoresis on a 8%-SDS-polyacrylamide gel. After separation, samples were transferred to polyvinylidene difluoride membranes (pore size, 0.45 μm; Amersham Biosciences, Freiburg, Germany) by Western blotting. Membranes blocked in 1% nonfat dry milk in a Tris-buffered saline/Tween 20 buffer were incubated at room temperature for 90 min with primary rabbit polyclonal ODC antibody (BIOMOL, Hamburg, Germany) recognizing human ODC diluted 1:1000 in Tris-buffered saline/Tween 20. Thereafter, the membranes were incubated with 1:3000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) for 1 h at room temperature. After incubation of the blots in substrate solution (Lumi-Light Western Blotting Substrate; Roche, Mannheim, Germany), the bands were visualized by chemiluminescence with the Lumi Imager (Roche).

Data Analysis. Data are means ± S.E.M. of n experiments. Statistical analysis were performed by Dunnett's test, if not stated otherwise.

Drugs Used. Agmatine sulfate, L-arginine, D-arginine, citrulline, and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma. Sodium nitroprusside was obtained from Schwarz Pharma (Monheim, Germany). N^1-Guanyl-1,7-diaminoheptane (GC7) was obtained from Biosearch Technology Inc. (Novato, CA). Drugs were dissolved in the respective media.

Results

Impact of Agmatine Application on Transcription and Translation of ODC in HepG2 Cells. The mRNA expressions of ODC and of the housekeeping gene β-actin were measured by means of quantitative PCR using sequence-specific primers and SYBR Green. Amplified products had melting curves indicating an amplification of the desired template (data not shown). The amount of mRNAs encoding ODC was approximately 2-fold higher in cells pretreated for 72 h with 1 mM agmatine than in untreated control cells ($n = 5$ in each series; $P < 0.0005$, t test for unpaired data).

After incubation of the cells with 1 mM agmatine for 72 h, no ODC protein could be visualized in Western blots (Fig. 1, A1–A5), whereas clear bands at 51 kDa were detected in control cells (no agmatine treatment; Fig. 1, C1–C5).

Antiproliferative Effect of Agmatine after Knock-Down of ODC-Antizyme-1 or Antizyme Inhibitor by RNA Interference in HepG2 Cells. Agmatine 1 mM inhibited cell proliferation (Fig. 2, left column) similarly as shown previously (Molderings et al., 2004). Transfection of HepG2 cells with siRNA against ODC-antizyme-1 or antizyme inhibitor did not change cell proliferation under control conditions [i.e., absence of agmatine (siRNA-targeting ODC-antizyme-1: 89.3 ± 5.1% of the corresponding cells without RNA interference, $n = 6$; siRNA targeting antizyme inhibitor: 93.8 ± 4.9% of the corresponding cells without RNA interference, $n = 5$; differences from 100% not statistically significant)]. In HepG2 cells transfected with siRNA targeting human ODC-antizyme-1, the antiproliferative effect of agmatine was significantly enhanced compared with nontransfected HepG2 cells (Fig. 2, left columns). In HepG2 cells transfected with siRNA targeting the human antizyme inhibitor protein, agmatine-induced inhibition of cell proliferation was not different from that of nontransfected HepG2 cells (Fig. 2).

Effect of L-Arginine and Citrulline on Agmatine-Induced Inhibition of Cell Proliferation in HT29 and HepG2 Cells. Agmatine 1 mM inhibited proliferation of the human colonic carcinoma cells HT29 on the average by 28 ± 2% ($n = 30$; averaging all respective data) compared with the corresponding controls. L-Arginine 1 mM and citrulline 1 mM also inhibited proliferation of HT29 cells by 24 ± 2 and 29 ± 2%, respectively (Fig. 3A), whereas 1 mM D-arginine was ineffective (102.5 ± 3.0% of the corresponding controls; $n = 7$). The inhibitory effect of 1 mM agmatine was not modified by coadministration with 1 mM L-arginine, 1 mM citrulline (Fig. 3A), or 1 mM D-arginine (inhibition of cell proliferation in the absence and presence of 1 mM D-arginine, respectively: 84.8 ± 2.1 and 85.9 ± 2.7%; $n = 3$–8).

Proliferation of the HepG2 Cells was inhibited by 1 mM agmatine on average by 37 ± 1% ($n = 11$; averaging all respective data shown in Figs. 3 and 4) compared with the corresponding controls. The data obtained with the human hepatoma cell line resembled those of the HT29 cells in that cell proliferation was also inhibited by 1 mM L-arginine (17 ± 1%; Fig. 3B) and 1 mM citrulline (21 ± 1%; Fig. 3B), respectively, and that the inhibitory effect of agmatine remained unchanged by coadministration of 1 mM L-arginine and 1 mM citrulline (Fig. 3B).

To investigate whether the antiproliferative effect of L-arginine and citrulline was due to a transformation of the compounds into agmatine by arginine decarboxylase (ADC), we determined their effect on the proliferation of HepG2 cells in which ADC was knocked down by RNA interference. Transfection with siRNA targeting human ADC did by itself not change proliferation of the HepG2 cells (95.2 ± 4.9% of the corresponding control cells without transfection; $n = 8$). After knockdown of ADC, 1 mM L-arginine and 1 mM citrulline failed to inhibit cell proliferation (Fig. 2, right columns). In control HepG2 cells, mRNA encoding ADC was detected in low quantity (only approximately 0.6% of the expression level

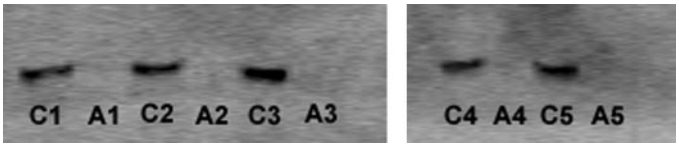

Fig. 1. Immunoblot of ornithine decarboxylase protein in HepG2 cells grown in the absence (C1–C5) or presence of 1 mM agmatine (A1–A5) for 72 h. Bands for the ornithine decarboxylase protein were visualized at approximately 51 kDa.

of mRNA encoding ODC-antizyme-1 or antizyme inhibitor), whereas in cells transfected with the siRNA, mRNA content was lower than the detection limit.

Experiments on the Potential Involvement of eIF5A in Agmatine-Induced Inhibition of Cell Proliferation in HT29 and HepG2 Cells. The selective potent inhibitor of deoxyhypusine synthase GC7 (Jakus et al., 1993), 1 μM given alone for 72 h had no effect on the proliferation of both cell lines (Fig. 4). The antiproliferative effect of 1 mM agmatine on HT29 and HepG2 cells was not altered by 1 μM GC7 (Fig. 4). Similar results were obtained when 1 μM GC7 was present in the culture medium for 7 days. In HT29 cells, proliferation in the presence of 1 μM GC7 was 100 $\pm$ 3.3% of that in its absence; the inhibitory effect of 1 mM agmatine in the presence of 1 μM GC7 was 99.7 $\pm$ 3.4% of that in its absence ($n = 5$). In HepG2 cells, proliferation in the presence of 1 μM GC7 was 104.3 $\pm$ 5.8% of that in its absence; the inhibitory effect of 1 mM agmatine in the presence of 1 μM GC7 was 91.7 $\pm$ 6.1% of that in the absence of GC7 ($n = 5$).

Interaction Experiments of Agmatine, L-Arginine, and Citrulline with L-NAME and Sodium Nitroprusside in HT29 Cells. The NO-synthase inhibitor L-NAME at a concentration of 100 μM inhibited proliferation of HT29 cells by 30 $\pm$ 2% (averaging all respective data shown in Fig. 5). The concentration of 100 μM L-NAME was chosen because it had been reported that L-NAME at that concentration inhibited NO-synthases in HT29 cells by approximately 50% (Blachier et al., 1995). The antiproliferative effect of 1 mM agmatine on HT29 cells was not affected by coadministration of 100 μM L-NAME (Fig. 5A). In addition, L-NAME 100 μM was also without influence on L-arginine- and citrulline-induced inhibition of HT29 cell proliferation (Fig. 5B). Sodium nitroprusside, which is known to generate NO nonenzymatically in HT29 cells (Blachier et al., 1996), inhibited proliferation of HT29 cells concentration-dependently (Fig. 6). The antiproliferative effect of 1 mM agmatine on HT29 cells was not affected by coadministration of 1 μM sodium nitroprusside; sodium nitroprusside 10 μM given on top of agmatine 1 mM caused an additional inhibition ($P < 0.05$; Fig. 6).

Determination of Agmatine-Induced Apoptosis. To investigate whether agmatine-induced inhibition of cell proliferation was due to apoptosis, we determined the activity of the apoptosis-related protein caspase-3 (Dlamini et al., 2004) in the human and rat hepatoma cell lines HepG2 and McRH7777, respectively, the human colon carcinoma cell line HT29 and the rat pheochromocytoma cell line PC-12. The antiproliferative effect of 1 mM agmatine was paralleled by an increase in caspase-3 activity in the four cell lines investigated (Fig. 7). The agmatine-induced increase in caspase-3 activity was clearly more pronounced in the rat cell lines McRH7777 and PC-12 compared with the human cell lines HepG2 and HT29 (Fig. 7).

Discussion

The aim of the present study was to challenge not yet examined potential intracellular sites and mechanisms of action that might underlie agmatine's antiproliferative effect on tumor cells. At the intracellular level, inhibition of cell proliferation by agmatine is associated with a reduction of

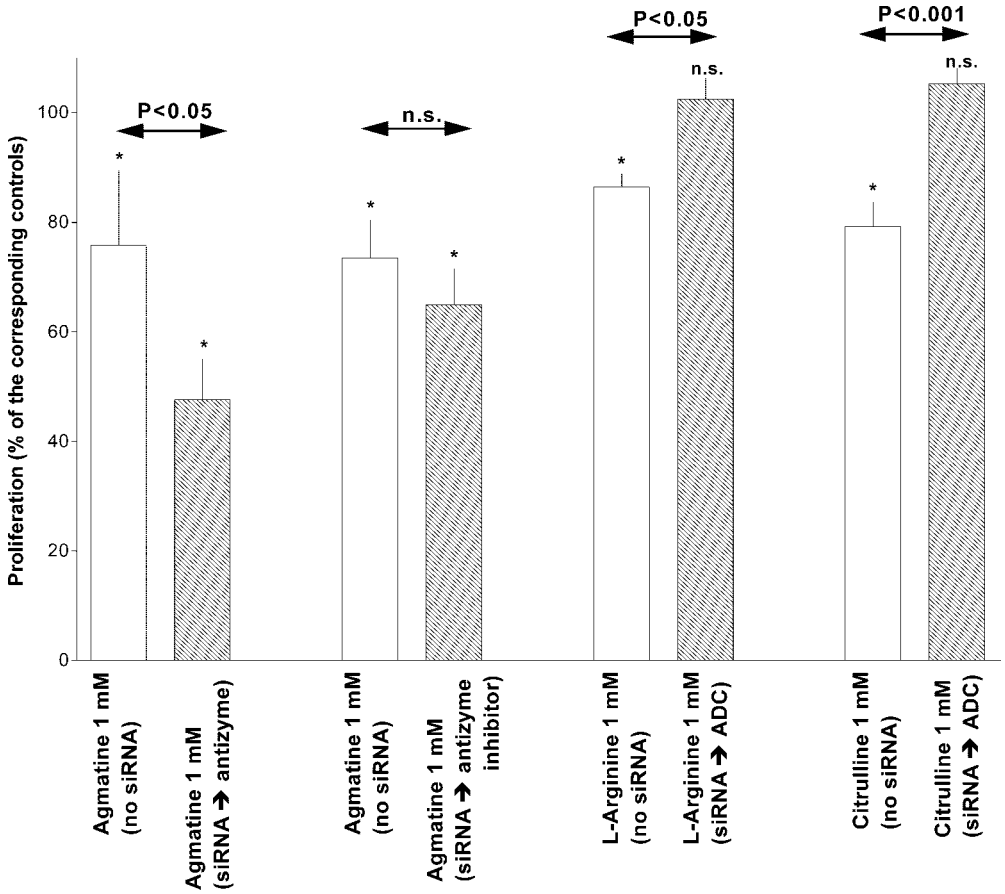

Fig. 2. Effects of 1 mM agmatine, 1 mM L-arginine, or 1 mM citrulline on the proliferation of HepG2 cells in untreated cells and cells transfected with siRNA targeting human ODC-antizyme-1 (antizyme), antizyme-inhibitor, or arginine decarboxylase (ADC), respectively. Cell number was estimated by measuring protein content. Cells were analyzed 4 days after transfection. Ordinate, cell proliferation expressed as a percentage of proliferation of nontransfected HepG2 cells in the absence of the test compounds. Mean $\pm$ S.E.M. of five to eight experiments in each series. *, $P < 0.05$ (compared with the corresponding controls in the absence of agmatine, L-arginine, or citrulline, respectively; Dunnett's test); Arrows, comparison of the proliferation under the conditions indicated below the columns (n.s., not significant; Tukey's test).

the intracellular levels of the polyamines (Satriano et al., 1998; Choi and Cho, 1999; Vargiu et al., 1999; Babal et al., 2001; Dudkowska et al., 2003; Higashi et al., 2004; Mayeur et al., 2005). ODC-antizyme induction has been supposed to be the main mechanism underlying agmatine's action, because negative regulation of the polyamine forming enzyme ornithine decarboxylase by agmatine was found to be paralleled by an induction of ODC-antizyme in some cell lines and tissues (Dudkowska et al., 2003; Gardini et al., 2003; Higashi et al., 2004). However, antizyme protein did not contribute to agmatine-induced down-regulation of ODC content in rat and human hepatoma cells (McRH7777, HepG2; Kribben et al., 2004), human colon carcinoma cells (HT29; Molderings et al., 2004), in the kidney of Swiss female mice (Dudkowska et al., 2003), and in rat hepatocytes (Vargiu et al., 1999). Thus, the imperative of antizyme protein in agmatine-induced decay of ODC activity and inhibition of polyamine uptake is questionable. Therefore, we investigated in the present study in HepG2 cells whether knockdown of ODC-antizyme-1 or antizyme inhibitor by means of RNA interference influences agmatine-induced inhibition of cell proliferation. Reduction of the ODC-antizyme-1 or antizyme inhibitor mRNAs did not significantly affect the proliferation of the cells after 72 h of treatment. When ODC-Az was knocked down in HepG2 cells, the antiproliferative effect of agmatine was enhanced. This is in contrast to the effect that one would expect if inhibition of ODC activity by agmatine would be due to an induction of ODC-Az. In that case, knockdown of ODC-Az should have abolished the inhibitory effect of agmatine. Silencing of the antizyme inhibitor gene did not affect agmatine's antiproliferative effect. Similar results were obtained in the rat hepatoma cell line McRH7777 (results not shown). Thus, our data argue against the contention that induction of ODC-antizyme is an imperative prerequisite for inhibition of cell proliferation by agmatine.

As shown by Western blotting, agmatine-induced inhibition of cell proliferation was accompanied by the abolition of ODC protein content in HepG2 cells, a phenomenon that has been reported previously for other cells (see above). Because the degradation of ODC by ODC-antizyme in the present experiments has been ruled out for HepG2 cells as the reason for the agmatine-induced reduction of ODC protein content and, because the mRNA content for ODC was increased (present study) or has been found to remain unchanged by agmatine (Dudkowska et al., 2003), we conclude that ODC is regulated by agmatine at the translational level. Tight regulation of ODC at the translational level linked to the intracellular polyamine content has been shown in addition to its posttranslational regulation by ODC-antizyme (Kameji and Pegg, 1987; Holm et al., 1989). The conflicting findings with

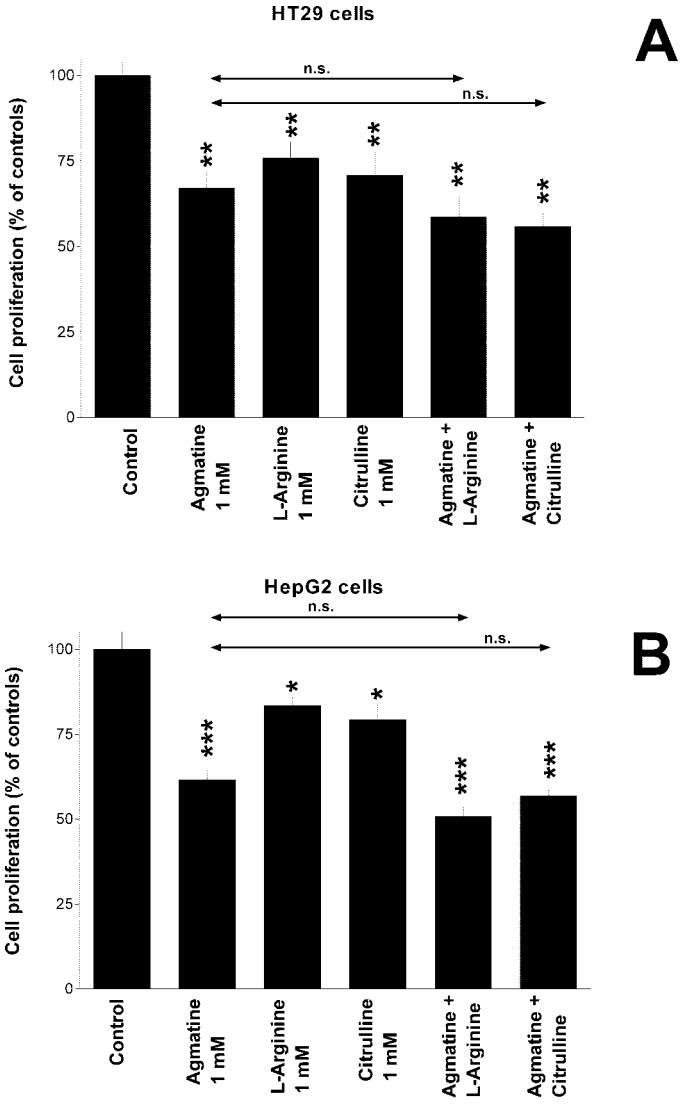

Fig. 3. Proliferation of HT29 cells (A) and HepG2 cells (B) in the absence and presence of 1 mM L-arginine, 1 mM citrulline, and/or 1 mM agmatine in the culture medium as indicated below the columns. The cells were incubated in the absence (controls) or presence of the drugs under study for 72 h. Cell number was estimated by determination of protein content. Ordinate, cell proliferation expressed as a percentage of proliferation of the respective cells in the absence of drugs. Mean ± S.E.M. of nine experiments (A) and five experiments (B), respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the corresponding controls; Dunnett's test); arrows, comparison of the proliferation under the conditions indicated below the columns (n.s., not significant; Tukey's test).

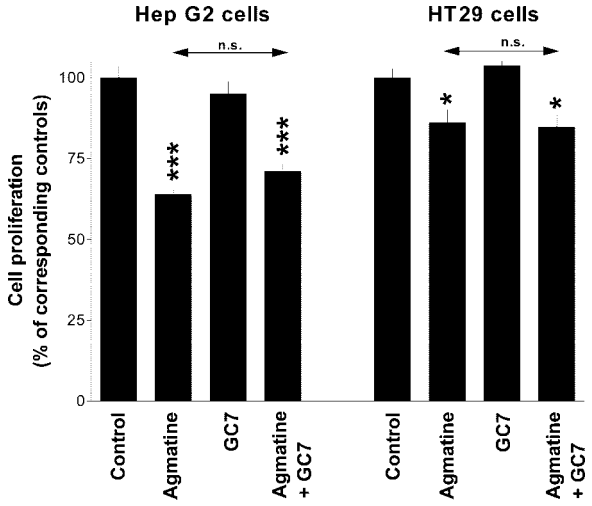

Fig. 4. Proliferation of HepG2 and HT29 cells in the absence and presence of 1 mM agmatine and/or 1 μM GC7 in the culture medium as indicated below the columns. The cells were incubated in the absence (control) or presence of the drugs under study for 72 h. Cell number was estimated by determination of protein content. Ordinate, cell proliferation expressed as a percentage of proliferation of the respective cells in the absence of drugs. Mean ± S.E.M. of six experiments in each series. *, $P < 0.05$; ***, $P < 0.001$ (compared with the corresponding controls; Dunnett's test); arrows, comparison of the proliferation under the conditions indicated below the columns (n.s., not significant; Tukey's test).

respect to the induction of ODC-antizyme by agmatine may be explained by the possibility that agmatine behaves as a partial agonist at the sites of action of the polyamines. If agmatine is applied when the intracellular levels of polyamines and in consequence the ODC-antizyme-1 content are high, agmatine may reduce both polyamine levels and ODC-antizyme content because regarding the sites and mechanisms responsible for the expression of ODC-antizyme, it cannot fully substitute for the reduced polyamine content. However, if agmatine is administered when polyamine level is low, which is associated with a very low ODC-antizyme content, agmatine may be hypothesized to further reduce the polyamine content on the one hand but on the other hand stabilize or slightly increase ODC-antizyme concentration by its supposed own partial agonistic action at the sites involved in the regulation of the expression of ODC-antizyme.

It was conceivable that agmatine might reduce the availability of L-arginine for ornithine synthesis by arginase, thus leading to a reduction of the content of ornithine (i.e., the substrate for putrescine formation by ODC). However, both L-arginine and citrulline failed to antagonize the antiproliferative effect of agmatine (Fig. 3) when added to the culture medium (which already contains L-arginine at concentrations of approximately 0.5 mM in Dulbecco's modified Eagle's medium and 1.1 mM in RPMI 1640 medium). It is interesting that when given alone, L-arginine but not D-arginine, as well as citrulline inhibited cell proliferation by themselves (Fig. 3). The low amount of nitric oxide originating from L-arginine in these cells (Blachier et al., 1995) can hardly be involved in the inhibition of cell growth by L-arginine or citrulline, because inhibition of nitric-oxide synthase by L-NAME did not affect this inhibitory effect (Fig. 5B). However, L-arginine or citrulline-induced inhibition of cell growth was abolished after reduction of the mRNA encoding arginine decarboxylase by the RNA interference technique (Fig. 2). Hence, in the human hepatic and intestinal tumor cells investigated citrulline is probably converted into L-arginine (Selamnia et al., 1998), which in turn is transformed into agmatine by constitutively expressed ADC (present study).

A further hypothesis that has not yet been challenged experimentally was that the antiproliferative effect of agmatine might to some extent be due to interactions with the NO system (Satriano 2004). NO has been reported to stimulate

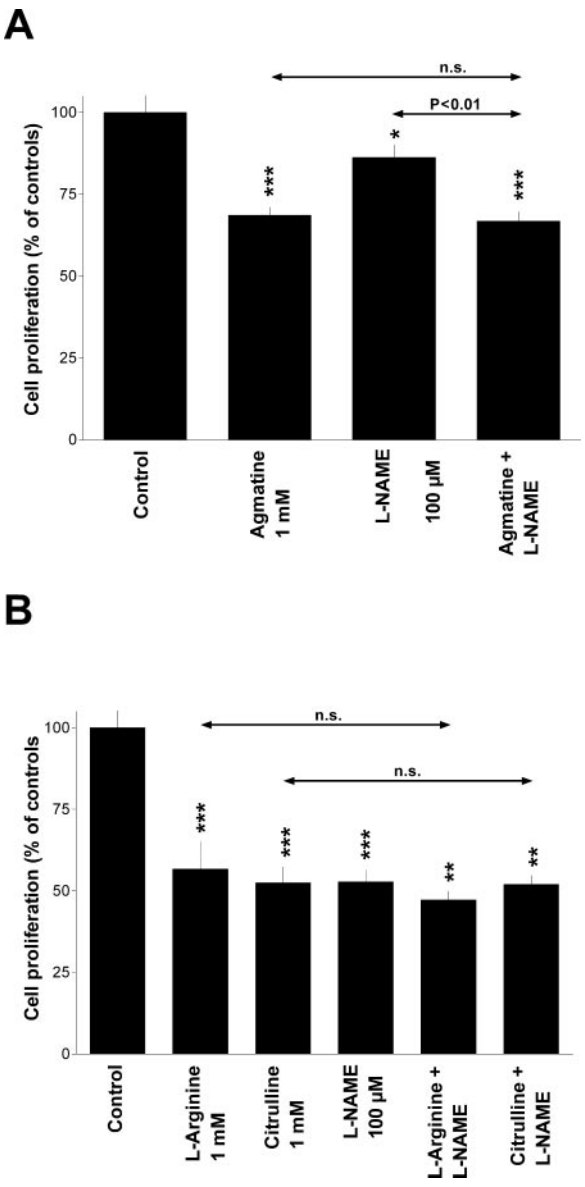

Fig. 5. A, proliferation of HT29 cells in the absence and presence of 1 mM agmatine and/or 100 μM L-NAME in the culture medium as indicated below the columns. B, proliferation of HT29 cells in the absence and presence of 1 mM L-arginine, 1 mM citrulline, and/or 100 μM L-NAME in the culture medium as indicated below the columns. The cells were incubated in the absence (control) or presence of the drugs under study for 72 h. Cell number was estimated by determination of protein content. Ordinate, cell proliferation expressed as a percentage of proliferation of the respective cells in the absence of drugs. Mean ± S.E.M. of six experiments in each series. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with the corresponding controls; Dunnett's test); arrows, comparison of the proliferation under the conditions indicated below the columns (n.s., not significant; Tukey's test).

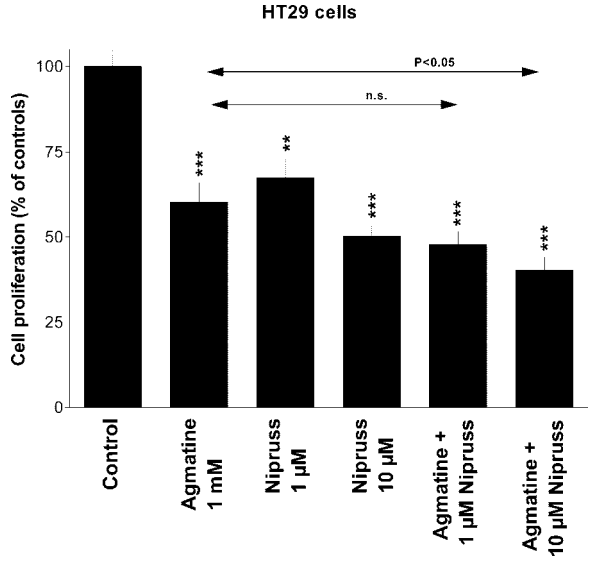

Fig. 6. Proliferation of HT29 cells in the absence and presence of 1 mM agmatine and/or 1 μM or 10 μM sodium nitroprusside (Nipruss) in the culture medium as indicated below the columns. The cells were incubated in the absence (control) or presence of the drugs under study for 72 h. Cell number was estimated by determination of protein content. Ordinate, cell proliferation expressed as a percentage of proliferation of the respective cells in the absence of drugs. Mean ± S.E.M. of six experiments in each series. **, $P < 0.01$; ***, $P < 0.001$ (compared with the corresponding controls; Dunnett's test); arrows, comparison of the proliferation under the conditions indicated below the columns (n.s., not significant; Tukey's test).

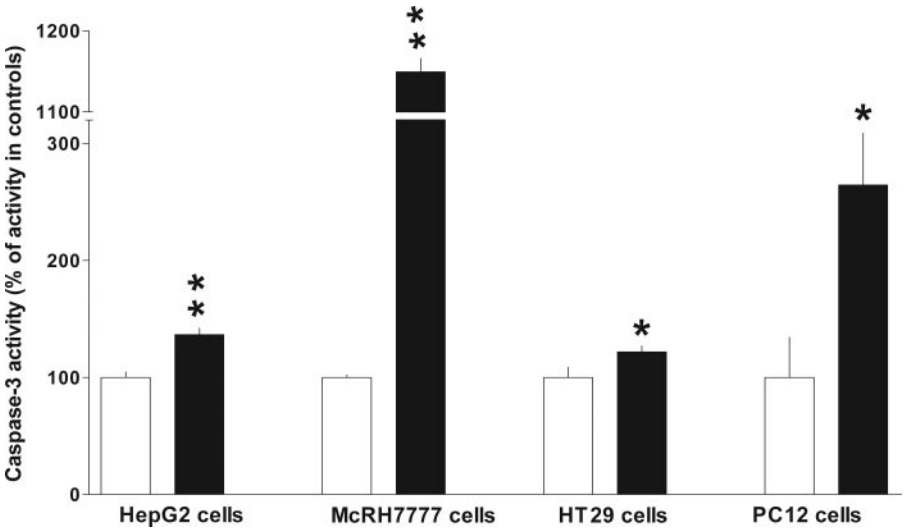

Fig. 7. Influence of 1 mM agmatine on apoptosis in HepG2 cells, McRH7777 cells, HT29 cells, and PC-12 cells (■). Apoptosis in controls (i.e., in the absence of agmatine, □). The cells were incubated in the absence (controls) or presence of the drugs under study for 72 h. Apoptosis was estimated by determination of the level of intracellular caspase-3 activity. The results are expressed as percentage of caspase-3 activity in the absence of agmatine. Mean ± S.E.M. of four to six experiments in each series. *, $P < 0.05$; **, $P < 0.01$ (compared with the corresponding controls; t test).

cell growth and protect many cell types from apoptosis at low intracellular concentrations, whereas high concentrations of NO can inhibit cell growth and induce apoptosis (Blachier et al., 1996; Siegert et al., 2002; Liu et al., 2003). The inhibitor of NO-synthase L-NAME at a concentration of 100 μM, which efficiently inhibits HT29 cell NO synthase activity by approximately 50% (Blachier et al., 1995), reduced proliferation of the HT29 cells by approximately 30% (Fig. 5), indicating that the low concentration of NO generated from L-arginine (Blachier et al., 1995) is in fact involved in promoting growth of HT29 cells. In contrast, when exposed to the NO donor sodium nitroprusside HT29 cell proliferation was concentration-dependently inhibited (Fig. 6), which is in accordance with previous finding in this cell line (Blachier et al., 1996). Finally, interaction experiments with agmatine and L-NAME or sodium nitroprusside revealed that at least in HT29 cells, agmatine's antiproliferative effect is not essentially due to an alteration of the intracellular NO level by agmatine.

We hypothesized that the antiproliferative effect of agmatine might be due, at least in part, to a distinct decrease in the intracellular content of eIF5A as a consequence of the agmatine-induced decrease in cytosolic spermidine concentration. In the present experiments, 1 μM concentration of the potent inhibitor of deoxyhypusine synthase GC7 (Jakus et al. 1993) failed to inhibit proliferation of HT29 and HepG2 cells (Fig. 4), suggesting that in these cells, eIF5A is not pivotal for growth. Moreover, GC7 did not influence the antiproliferative action of agmatine (Fig. 4), ruling out a dependence of agmatine's action on eIF5A activity for both cell lines. This conclusion is in agreement with recent data, which showed the critical requirement of polyamines in mammalian cell proliferation to be independent of hypusine synthesis (Nishimura et al., 2005).

Because the decrease of intracellular polyamine content has been shown to correlate with the progression of apoptosis (Pignatti et al., 2004), we investigated whether agmatine can promote apoptosis. Induction of programmed cell death by agmatine has been described previously in isolated rat hepatocytes (Gardini et al., 2001). However, only a cytostatic effect of agmatine without induction of apoptosis has been observed in transformed NIH/3T3 fibroblasts (Isome et al., 2003) and the rat hepatoma cells HTC and JM2 (Gardini et al., 2003). The present experiments, in which we determined caspase-3-like activity as a marker of apoptosis (Dlamini et al., 2004), revealed that agmatine induced apoptosis in the four tumor cell lines under study in such a way that the higher the degree of caspase-3-like activity, the higher the inhibition of cell proliferation. Agmatine-induced increase in caspase-3 activity was clearly more pronounced in the rat cell lines McRH7777 and PC-12 compared with the human cell lines HepG2 and HT29 (Fig. 7), suggesting species differences in the susceptibility to the apoptosis-inducing property of agmatine.

In conclusion, agmatine administration to tumor cells in vitro induces a decrease of intracellular polyamine levels because, as a result of its structural similarity, it interferes as a partial agonist with their metabolism. The data from the in vitro experiments in cell lines are compatible with the idea that in vivo, a decrease in intracellular agmatine concentration might be associated with neoplastic growth (Molderings et al., 2004) (i.e., agmatine serves as a regulatory component of the polyamine pathway) (Molderings, 2006).

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