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ORIGINAL ARTICLE

Chronic exposure to agmatine results in the selection of agmatine-resistant hepatoma cells

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Abstract During our study of the cytostatic effect of agmatine, we were able to isolate an agmatine resistant clone from a parental hepatoma cell line, HTC. These cells, called Agres, had slower growth rate than the parental cells when cultured in normal medium. The modification in polyamine content induced by agmatine was much lower in these cells and ornithine decarboxylase, S-adenosylmethionine decarboxylase and spermidine/spermine acetyltransferase activities were much less affected. By investigating the mechanism responsible for these modifications, it was shown that agmatine and polyamines were not taken up by Agres cells. Their resistance to the antiproliferative effects of agmatine may thus arise from a lack of the polyamine transport system. Moreover, Agres cells were able to take up both glutamic acid and arginine at a rate significantly higher than that detected for HTC cells, most likely to provide components for compensatory increase of PA synthesis. These results emphasize the importance of polyamine transport for cell growth.

Keywords Agmatine · Polyamines · Uptake · Glutamic acid · Arginine · HTC cells

Abbreviations

DFMO	Difluoromethylornithine
MGBG	Methylglyoxal bisguanylhydrazone
ODC	Ornithine decarboxylase
PUT	Putrescine
SAMDC	S-adenosylmethionine decarboxylase
SPD	Spermidine
SPM	Spermine
SSAT	Spermidine/spermine acetyltransferase
HTC	Rat hepatoma cells
PA	Polyamines
ECL	Enhanced chemiluminescence
Agres	Agmatine resistant cell line

Introduction

In mammalian cells, polyamines (PA, putrescine, spermidine and spermine) are essential for proliferation, differentiation and neoplastic transformation (Pegg 1986; Jänne et al. 2004; Casero and Marton 2007; Agostinelli et al. 2010). Their cellular content is carefully regulated by a delicate equilibrium depending on changes in activity of synthetic and interconverting enzymes, as well as on the control of their uptake and release from the cell. Several laboratories have provided efforts designed to modulate their concentration involving the use of specific inhibitors of PA synthesis such as difluoromethylornithine (DFMO), known to be active on ornithine decarboxylase (ODC) (McCann and Pegg 1992), or methylglyoxal bisguanylhydrazone (MGBG) (Pegg and McCann 1992) and its analogs

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(among these 4-amidinoindane-1-one-2′-amidinohydrazone, CGP 48664A), active on S-adenosylmethionine decarboxylase (SAMDC) (Regenass et al. 1992). Polyamine analogs that down-regulate ODC and up-regulate spermidine/spermine acetyltransferase (SSAT), such as N^1,N^{11}-diethylnorspermine (Porter et al. 1991), have also been employed. However when these agents were evaluated in clinical trials, results were not completely satisfactory (Wolff et al. 2003). Further work is therefore required to evaluate the possibility of utilizing polyamine analogs alone or in combination with cytotoxic drugs in trials designed for cancer therapy.

Another polycationic molecule, structurally analogous to PA, is agmatine. Agmatine is formed by the decarboxylation of arginine that, originally believed to be formed only in bacteria, plants and invertebrates (Tabor and Tabor 1984), has been now detected in mammalian tissues (Raash et al. 1995). It has since been shown to promote several different effects (Reis and Regunathan 2000; Grillo and Colombatto 2004; Battaglia et al. 2010a, b).

Previous studies from our laboratory on agmatine metabolism and effects have demonstrated that this molecule is taken up by hepatocytes through the same carrier that also recognizes the PA putrescine, and is then partially degraded by diamine oxidase (Cabella et al. 2001). Increased intracellular levels of agmatine are able to down regulate ODC activity as well as to increase expression and activity of SSAT, leading to a very significant decrease in the intracellular concentration of polyamines (Vargiu et al. 1999). These modifications can promote either caspase-dependent apoptosis in non-growing cells like cultured rat hepatocyte or cell cycle arrest (G_2/M) in rapidly proliferating cells such as hepatoma cells (Gardini et al. 2001, 2003).

In this study, we designed experiments to assess whether chronic exposure of hepatoma cells to agmatine, a putative anti-cancer agent already used in vitro studies, results in the induction of resistance, as observed for other polyamine analogs. Indeed, long term (i.e., several months) exposure of rat liver hepatoma cells (HTC) to increasing agmatine concentrations led us to isolate a clone of agmatine resistant (Agres) cells and to identify the related mechanism enabling these cells to overcome the growth inhibitory effect of millimolar agmatine.

Materials and methods

Materials

L-[1-^{14}C]-ornithine, [1-^{14}C]-acetylCoA, L-[U-^{14}C]-arginine, S-[1-^{14}C]-adenosylmethionine, [U-^{14}C]-putrescine, [U-^{14}C]-spermidine, [U-^{14}C]–spermine, L-[U-^{14}C]-glutamic acid, horseradish peroxidase-conjugate anti-rabbit IgG and anti-mouse Cy3-conjugated secondary antibody were from GE Healthcare Europe GmbH (Cologno Monzese, Italy). Horse serum and penicillin/streptomycin were from SPA (Milano, Italy). Monoclonal antibody anti-α tubulin and all other chemicals were from Sigma Chemical Co (St Louis, MO, USA).

Cell culture and isolation of an agmatine-resistant clone (Agres) from HTC cells

HTC cells (rat liver hepatoma cells derived from Morris hepatoma; ECACC, Salisbury, UK) were routinely grown as a monolayer in RPMI 1640 medium supplemented with 10% (v/v) horse serum and containing 100 U penicillin and 100 μg/ml streptomycin. After attachment overnight, the medium was changed and the cells were incubated as described for the desired time.

Agres cells were selected for agmatine tolerance by growing HTC cells in a culture medium containing stepwise increasing concentrations of the polyamine analog. Initially, the medium contained 10 μM agmatine and every 5 days the concentration was doubled to finally reach 10 mM. This resulted in the selection of a clone of surviving agmatine-resistant cells (Agres) that were able to grow in the presence of 10 mM agmatine.

Cell growth was measured by crystal violet staining in cells grown in 96-well flat-bottomed microplates, as previously described (Vargiu et al. 1999).

Synthesis of [^{14}C]- agmatine

Synthesis and purification of [^{14}C]-agmatine were performed as previously described (Cabella et al. 2001). Briefly, 18 mM [L-^{14}C-U]-arginine (0.6 mCi/mmol^{-1}) in 0.2 M sodium acetate (pH 5.2), 5 mM pyridoxal phosphate and 0.1% BSA, was treated with *Escherichia coli* arginine decarboxylase (1 I.U.) and incubated at 37°C. After 2 h fresh enzyme was added and incubation was continued for another 3 h. The reaction was stopped by addition of 5 mM KOH in a NaCl saturated solution and agmatine was extracted with *n*-butanol. Radiochemical purity of agmatine was checked by HPLC after derivatization with *o*-phtalaldehyde.

Assays

All experiments were performed on sub-confluent (70%) parental HTC and Agres cells that were cultured for two or more passages in agmatine-free medium.

Analysis of the uptake of [^{14}C]-compounds has been described by Cabella et al. (2001); assay of ODC, SAMDC and SSAT activities using [^{14}C]-substrates, as well as HPLC determination of polyamines after dansylation were performed as described by Vargiu et al. (1999).

Western blot of ODC was performed as previously described by Gardini et al. (2003). Briefly, the cells were collected by boiling Laemmli sample buffer. After sonication and centrifugation aliquots of the supernatant (100 μg protein) were resolved electrophoretically on 12% SDS–PAGE. Protein was measured as described by Lowry et al. (1952). Immunoblotting of ODC was carried out with a specific rabbit polyclonal antiserum prepared in our laboratory. The ODC-antibody complexes were detected by ECL using a goat anti-rabbit IgG linked to horseradish peroxidase.

Immunofluorescence detection of α-tubulin

HTC and Agres cells were washed with PBS and fixed with methanol-acetone (1:1 v/v) for 15 min at −20°C. After rehydratation with PBS containing 0.1% Triton X-100 and 1% bovine serum albumin, cells were incubated with monoclonal anti-α-tubulin (1:1000) at 4°C overnight followed by detection with anti-mouse Cy3-conjugated secondary antibody and nuclei counterstaining with Hoechst 33258.

Statistical analysis

Data presented are the mean ± SEM of at least three independent experiments.

Results

Long term exposure of HTC cells to increasing concentrations of agmatine, as described under "Materials and methods", resulted in the selection of a clone of cells tolerant to the persisting presence of 10 mM agmatine in the culture medium (Agres cells). When cultured in normal medium, Agres cells were characterized by a growth rate slower than that of parental HTC cells. Accordingly, addition of agmatine at a concentration (0.5–2.0 mM) that abolished proliferation of parental HTC cells was completely ineffective on Agres cells (Fig. 1).

The antiproliferative effect of agmatine, operated by depleting parental cells of endogenous polyamines (Table 1), as previously reported (Gardini et al. 2003). The analysis of intracellular level of polyamines in Agres cells showed a threefold increase in putrescine and a twofold increase in spermine when compared with parental HTC; however, the addition of agmatine had a negligible effect on both, resulting only in a decrease of putrescine (Table 1).

The agmatine-promoted depletion of polyamines in HTC parental cells was the result of a dramatic reduction of ODC activity and a significant induction (approx. eightfold) of SSAT activity. Agmatine exposure resulted only in a twofold induction of SAMDC activity in parental cells (Table 2). These changes were markedly attenuated in Agres cells. It should be noted that the marked reduction of ODC activity in parental HTC cells was paralleled by an agmatine-dependent reduction in ODC protein levels. This effect was not observed in Agres cells (Fig. 2).

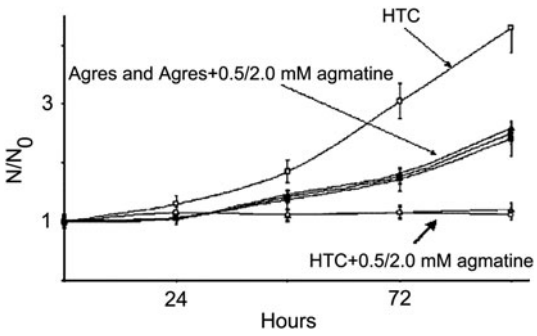

Fig. 1 Agmatine effect on growth of parental HTC (*open symbols*) and agres cells (*filled symbols*). Cells were grown in the absence (*open square*, *filled square*) or presence of 0.5 (*open triangle*, *filled triangle*) or 2.0 (*open circle*, *filled circle*) mM agmatine as described in "Materials and methods". Growth is represented as the ratio of N/N_0, where N_0 is the original seeding density (1,000 cells/each well in 96-wells flat-bottomed microplates) and N is the cell number at the indicated times

Table 1 Polyamine content in parental HTC and Agres cells

	Addition	PUT	SPD	SPM
Parental cells (nmoles/mg protein)	–	0.23 ± 0.02	3.57 ± 0.42	3.36 ± 0.35
	Agmatine	0.20 ± 0.01	0.64 ± 0.04	0.03 ± 0.01
Agres cells (nmoles/mg protein)	–	0.68 ± 0.05	3.35 ± 0.37	7.43 ± 0.68
	Agmatine	0.15 ± 0.02	2.93 ± 0.26	6.79 ± 0.53

Effect of agmatine. Cells cultured for 48 h in the presence or absence of 2 mM agmatine were used to evaluate polyamine content as described in "Materials and methods"

Table 2 ODC, SAMDC and SSAT activitiy in parental HTC and Agres cells

Activity (nmoles/h/mg protein)				
	Addition	ODC	SAMDC	SSAT
Parental cells	–	9.6 ± 0.8	0.30 ± 0.02	0.31 ± 0.03
	Agmatine	0.3 ± 0.05	0.64 ± 0.04	2.55 ± 0.06
Agres cells	–	14.6 ± 1.2	0.28 ± 0.04	0.32 ± 0.03
	Agmatine	11.2 ± 1.0	0.39 ± 0.03	0.51 ± 0.03

Effect of agmatine. Cells cultured for 48 h in the presence or absence of 2 mM agmatine were used to measure the enzyme activities as previously described in "Materials and methods"

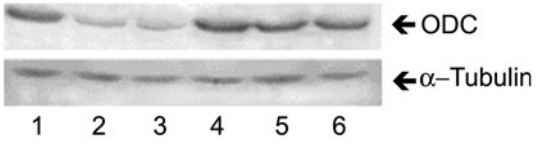

Fig. 2 Agmatine effect on ODC protein level. The cells were cultured for 48 h in the presence or absence of 2 mM agmatine. The blot is representative of three experiments. *1* parental cells, *2* parental cells treated for 48 h with 0.5 mM agmatine, *3* parental cells treated for 48 h with 2.0 mM agmatine, *4* Agres cells, *5* Agres cells treated for 48 h with 0.5 mM agmatine, *6* Agres cells treated for 48 h with 2 mM agmatine. α-tubulin was used as a loading control

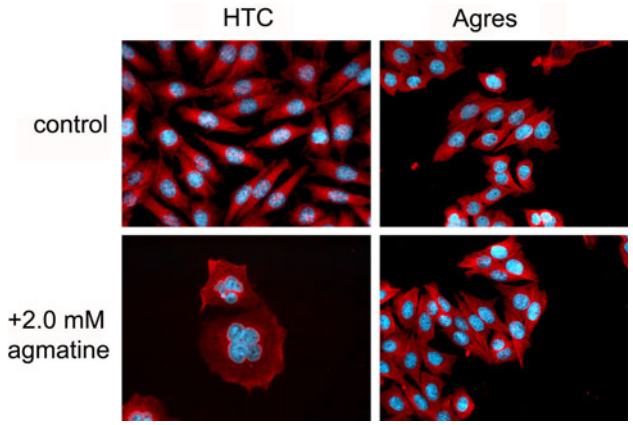

Fig. 3 Effect of agmatine on α-tubulin filaments organization in parental HTC and Agres cells. Cells were cultured for 72 h in presence or not of 2.0 mM agmatine. Microtubule network was labeled by α-tubulin staining with specific monoclonal antibody, followed by incubation with a second antibody-Cy3 coniugate. The nuclei were stained blue with Hoecht 33258

Changes in polyamine content and activity of polyamine-related enzymes induced by agmatine in HTC cells coincided with evident morphological alterations (Fig. 3), including changes in cytoskeletal structure (as exemplified by reorganization of α-tubulin), as well as the appearance of multinuclear cells. These striking morphological changes were not detected in Agres cells.

To investigate the mechanism leading to resistance in Agres cells, we first measured the ability to take up radiolabelled agmatine. As reported in Table 3, the uptake in Agres cells was extremely low if compared to the one detected in parental cells. Since it is well known that agmatine is taken up by the same transporter(s) used by polyamines, we then determined whether Agres cells were able to incorporate radiolabelled PA. When radiolabelled polyamines were used in the standard range of concentrations (from 0.25 to 45 μM, with parental HTC showing a saturable uptake), Agres cells were found to be unable to transport these compounds into the cell (Fig. 4).

To further confirm that Agres cells were resistant to agmatine as a consequence of a lack of a specific and

Table 3 Uptake of [^{14}C]-agmatine in parental HTC and Agres cells

	nmoles/h/mg protein
Uptake of [^{14}C]-agmatine	
Parental cells	2.70 ± 0.31
Agres cells	0.07 ± 0.01

Cells were grown for 72 h. The medium was then replaced with new medium containing 50 μM [^{14}C]-agmatine (1 μCi/μmole). After 60 min the cells were processed as described in "Materials and methods"

transporter(s)-mediated uptake, we designed experiments to evaluate the ability of putrescine to counteract DFMO-induced growth inhibition. As expected, this unequivocally occurred in parental HTC but not in Agres cells (Fig. 5), confirming that the latter cells were unable to take up putrescine.

The polyamine pool in Agres cells was preserved or even slightly increased relative to parental HTC cells, and were deficient in PA transport, yet maintained high ODC activity. We therefore evaluated the import of the components required for compensatory intracellular PA synthesis in these cells. Agres cells were shown to be able to take up both glutamic acid and arginine at a rate significantly higher than that detected for HTC cells (Fig. 6). Agres cells may thus circumvent the block of exogenous PA uptake by increasing the uptake of ornithine precursors required for PA biosynthesis.

Polyamines regulate their intracellular concentrations by inducing ODC-antizyme (AZ), which suppresses both ODC activity and PA transport. It is known that cycloheximide treatment abrogates the repression of polyamine uptake mediated by AZ (Lopez-Contreras et al. 2008). Therefore, we performed experiments to determine if the lack of polyamine uptake was due to AZ. Addition of 100 μM cycloheximide 3 h prior to loading Agres cells with [^{14}C]-putrescine had no effect on the AZ-regulated uptake of the polyamine ([^{14}C]-putrescine transport in Agres cells was 0.14 and 0.11 nmoles/hr/mg protein in control and cycloheximide-treated cells, respectively). The fact that putrescine transport rate was not increased by cycloheximide treatment induces to exclude that AZ is responsible for the lack of PA uptake in Agres cells.

Discussion

Agmatine is an ubiquitous molecule present in mammalian fluids and tissues, as well as in bacteria, plants and invertebrates. It can originate from absorption in the gastrointestinal tract and/or from the "intestinal flora", although its tissue distribution is poorly correlated with tissue blood flow, suggesting local synthesis by arginine decarboxylase

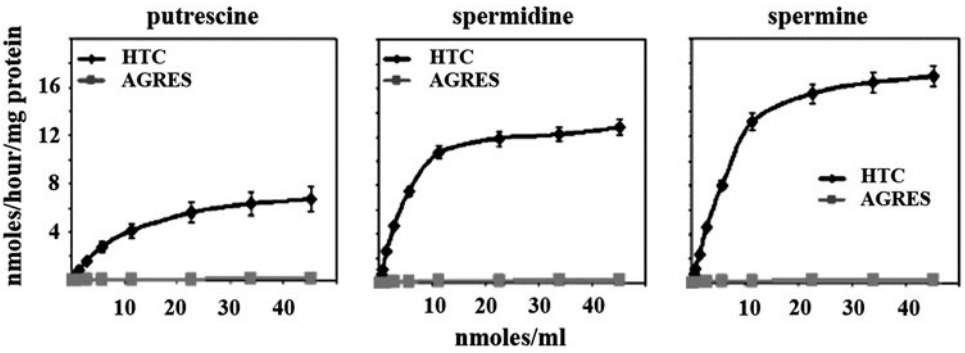

Fig. 4 Polyamine uptake by HTC and Agres cells. Putrescine, spermidine and spermine uptake activities in HTC (*filled circles*) and Agres cells (*filled square*) were measured as described in "Materials and methods"

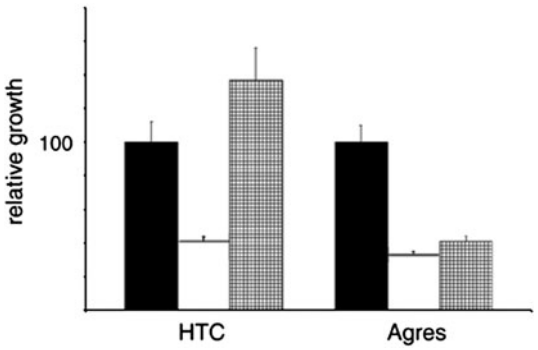

Fig. 5 Exogenous putrescine effect on cell growth in DFMO treated cells. Cells were seeded in 96-wells flat-bottomed microplates. Optical density relative to crystal violet staining was measured every 24 h. The figure shows the results obtained at 120 h expressed as the percentage of control cell growth; *filled square* no addition, *open square* 3 mM DFMO, *grid square* 3 mM DFMO, and 20 μM putrescine

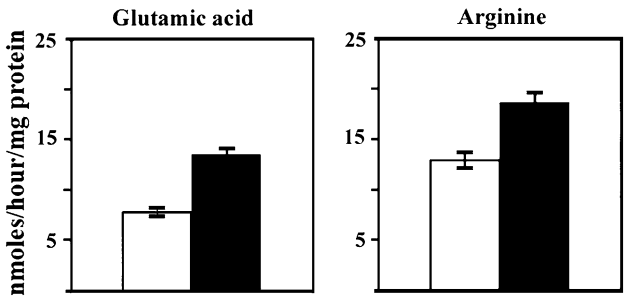

Fig. 6 Glutamic acid and arginine uptake by HTC and Agres cells. Glutamic acid and arginine uptake activities in HTC (*open square*) and Agres (*filled square*) cells were measured as described in "Materials and methods"

(Zhu et al. 2004) or by a still unknown pathway. Whatever the origin, agmatine can promote a broad spectrum of physiopathological effects, that are mediated, at least in part, by interaction with imidazoline type I_1, as well as I_2 (Piletz et al. 1995) and I_2 receptors (Li et al. 1994), and in part by interfering with several other pathways either directly or through its metabolites. In rat hepatocytes, a non-proliferating cell model, the same putative carrier used by putrescine takes up agmatine. Although a small amount of intracellular agmatine can be hydrolyzed to putrescine and urea by agmatinase (Cabella et al. 2001; Gardini et al. 2001; Satriano et al. 2001; Iyer et al. 2002; Mistry et al. 2002), agmatine principally accumulates as such or is catabolized by diamine oxidase to butyraldehyde and H_2O_2. Agmatine also decreases polyamine content by means of ODC down-regulation, inhibition of polyamine uptake, and overexpression of SSAT, the latter being able to induce a further increase of H_2O_2 by polyamine oxidase. H_2O_2, at least in cultured hepatocytes, can trigger agmatine-induced apoptosis (Gardini et al. 2001).

In transformed and rapidly growing cells, (HTC, HepG2, JM2) agmatine can act as a cytostatic agent and produces a progressive accumulation of cellular population in G_2/M phase as well as a dramatic alteration of cytoskeleton and morphology. The main target of agmatine seems to be polyamine content as spermidine can effectively reverse growth inibition (Gardini et al. 2003).

In view of these effects and on the basis of results provided by others (Satriano et al. 1998), it has been proposed that agmatine or its analogs could be used to modulate or prevent tumor growth. However, the development of agmatine resistance may represent a significant impediment to this approach, as already shown in the past for many chemotherapeutic drugs (Cukierman and Khan 2010). Here, we were able to develop an HTC clone (designated with the acronym Agres) that is able to survive and grow in the presence of 10 mM agmatine. Resistance to the cytostatic capacity of agmatine was permanent and it was preserved after many passages in agmatine free medium.

In Agres cells, resistance is neither linked to ODC gene mutations giving rise to a protein with longer half-life (data not shown) nor to the induction of multidrug resistant protein type 1 (MDR-1, as evaluated by Western blotting, data not shown). Agmatine tolerance is likely to represent a consequence of the failure to transport agmatine as well as other polyamine across the cell membrane (Table 3; Fig. 4). Moreover, exogenous putrescine counteracts DFMO- induced growth inhibition in HTC parental cells, but not in Agres cells (Fig. 5). The growth rate of Agres

cells is fourfold to fivefold slower than that of parental cells although their polyamine content is not decreased (Fig. 1; Table 1) suggesting that PA transport plays an important role in maintaining a normal cell growth rate.

In conclusion, we have selectively generated a hepatoma cell line that is resistant to the antiproliferative effects of agmatine which occurs due to a deficient PA–agmatine transporter. As the eukaryotic putative polyamine carrier(s) has not yet been isolated or cloned, this cell line may afford a new avenue for investigation of the complex mammalian polyamine transporter (Belting et al. 2003).

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