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Effects of agmatine accumulation in human colon carcinoma cells on polyamine metabolism, DNA synthesis and the cell cycle

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Abstract

Putrescine, spermidine and spermine are low molecular polycations that play important roles in cell growth and cell cycle progression of normal and malignant cells. Agmatine (1-amino-4-guanidobutane), another polyamine formed through arginine decarboxylation, has been reported to act as an antiproliferative agent in several non-intestinal mammalian cell models. Using the human colon adenocarcinoma HT-29 $Glc^{-/+}$ cell line, we demonstrate that agmatine, which markedly accumulated inside the cells without being metabolised, exerted a strong cytostatic effect with an IC50 close to 2 mM. Agmatine decreased the rate of L-ornithine decarboxylation and induced a 70% down-regulation of ornithine decarboxylase (ODC) expression. Agmatine caused a marked decrease in putrescine and spermidine cell contents, an increase in the N_1-acetylspermidine level without altering the spermine pool. We show that agmatine induced the accumulation of cells in the S and G_2/M phases, reduced the rate of DNA synthesis and decreased cyclin A and B_1 expression. We conclude that the anti-metabolic action of agmatine on HT-29 cells is mediated by a reduction in polyamine biosynthesis and induction in polyamine degradation. The decrease in intracellular polyamine contents, the reduced rate of DNA synthesis and the cell accumulation in the S phase are discussed from a causal perspective.

Keywords: Agmatine; Polyamine; Cell cycle; Cyclin A; Cyclin B_1; Metabolism

1. Introduction

Colonic epithelial cells are exposed to numerous dietary compounds and to related metabolites from both luminal and basolateral poles which may influence cell physiology [1,2]. Among them are polyamines, low molecular biogenic polycationic amines such as "classical" polyamines and agmatine, found in food from vegetal and animal origins and in fermented food products [3,4]. Polyamines are also produced by luminal colonic bacteria [5].

The "classical" polyamines (putrescine, spermidine, and spermine) are ubiquitous components of mammalian tissues and play essential roles in cell growth and differentiation [6,7]. Putrescine, the essential precursor of the higher polyamines, spermidine and spermine, is synthesised by the decarboxylation of ornithine by the key enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) [8]. Increased polyamine de novo synthesis is known to occur in rapidly proliferating cells including tumour cells [9]. Therefore, polyamine metabolism via the control of ODC protein activity and expression has for several years been considered as a target for chemotherapy [9–11]. An alternate

Abbreviations: ADC, arginine decarboxylase; BrdU, bromodeoxyuridine; DAO, diamine oxidase; DFMO, difluoromethylornithine; DMEM, Dulbecco modified Eagle medium; CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent-kinases inhibitors; FCS, fetal calf serum; HS, horse serum; LDH, lactate dehydrogenase; ODC, ornithine decarboxylase; PCA, perchloric acid; PI, propidium iodide; SAMDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N1-acteyltransferase; DENSPM, N1, N11-diethylnorspermine; N1A-Spd, N1-acetylspermidine; Put, Putrescine; Spd, spermidine; Spm, spermine; Agm, agmatine

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pathway to putrescine biosynthesis from L-arginine is well known to occur in bacteria and plants but is also found in mammals [12–15]. In mammals, it occurs via the direct decarboxylation of arginine through arginine decarboxylase (ADC EC 4.1.1.19) to produce agmatine, which is then converted into putrescine by agmatinase in different tissues including the brain, liver and kidney [12,16–19].

Agmatine is present in the lumen of the human gastrointestinal tract and stems from three sources [12]: (a) agmatine formed and released by colonic flora [5], (b) unabsorbed agmatine contained in variable amounts in ingested food [4,12,20], (c) luminal agmatine derived from desquamated intestinal epithelial cells. Among its numerous physiological roles [21–24], agmatine has been shown to interfere with polyamine metabolism using ex vivo and in vitro experiments [25–28] and with cell growth in non-intestinal tumour and healthy cells without exerting any toxic effects [25,29–32]. Moreover, exogenous agmatine is known to be absorbed by the gastrointestinal tract and strongly accumulated in the liver and intestine [33].

Thus, the aim of the present study was to document whether agmatine altered cell proliferation in two human colon adenocarcinoma epithelial cell lines, namely HT-29 and $CaCo_2$. Then, we characterised the agmatine effect on polyamine metabolism and on cell cycle distribution, DNA synthesis and cell cycle-regulated protein expression. In addition, we determined that agmatine strongly accumulated within colonic cells without being metabolised.

2. Materials and methods

2.1. Materials

Agmatine sulfate, which was purchased from Fluka (St Quentin Fallavier, France), contained no putrescine and only 1% spermine. Methylbenzethonium hydroxide was purchased from Fluka. L-Ornithine hydrochloride, bis-benzymide trihydrochloride (HOECHST33342), pyridoxal 5′-phosphate, and dithiotreitol were purchased from Sigma chemicals (St Quentin Fallavier, France). A protease inhibitor complete mini cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). L-[1-^{14}C] ornithine, [1,4-^{14}C] putrescine, L-[guanido-^{14}C] arginine and L-[1-^{14}C] glucose were purchased from New England Nuclear (Boston, MA, USA). [Guanido-^{14}C]agmatine was purchased from Bio Trend (Köln, Germany).

2.2. Cells and culture conditions

The HT-29 $Glc^{-/+}$ cells used in this study were kindly provided by Zweibaum et al. [34]. These human colon adenocarcinoma cells were selected from the parental line by growing them in a glucose-free medium for 36 passages and then switching them to a 25 mM D-glucose containing medium. These cells are known to accumulate and metabolise butyrate, which is the major energetic substrate of healthy colonic epithelial cells [35]. HT-29 $Glc^{-/+}$ cells were grown at 37 °C under 10% CO_2 atmosphere in a Dulbecco modified Eagle medium (DMEM) containing 4 mM L-glutamine, 25 mM D-glucose and supplemented with 10% fetal calf serum (FCS). $CaCo_2$ cells were cultured in the same medium except for the presence of 20% FCS and 1% non-essential amino acids. HT-29 $Glc^{-/+}$ cells were used from passage 40 to 71 and $CaCo_2$ cells were used from passage 101 to 127 (1 passage per week). Agmatine sulfate or equal amounts of H_2SO_4 (control) was always added 2 days after cell seeding in DMEM supplemented with 3% horse serum (HS) in order to avoid the polyamine degradation by serum amine and diamine oxidase (DAO) activities. Indeed, DAO is involved in agmatine and polyamine catabolism. Using the Okuyama and Kobayashi method [36], we found that horse serum contained less DAO activity than FCS (0.37 ± 0.11 pmol/μl/h versus 0.88 ± 0.10 pmol/μl/h) so that the diamine oxydase present in the cultured medium supplemented with 3% HS was reduced eight fold in comparison with the culture medium containing 10% FCS. H_2SO_4 controls were first maintained under 10% CO_2 atmosphere in order to reach a neutral pH before the experiments. To test the long-term effect on cell proliferation, the media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml amphotericin B. The media were changed every day.

2.2.1. Cell proliferation

HT-29 $Glc^{-/+}$ and $CaCo_2$ cells were seeded at a density of 5×10^3 cells per well on 96-well tissue culture microplates (Costar, Marne la Vallée, France) and cell proliferation was determined using the method of DNA fluorometric assay using bis-benzimide trihydrochloride i.e. Hoechst 33342 [37]. Fluorescence intensity was quantified (λex=360 nm, λem=465 nm) using the microplate cytofluorometer Spectrafluorplus (TECAN, Trappes, France). The results were expressed as the number of cells per well using a linear standard curve.

2.2.2. Membrane integrity

Membrane integrity was estimated by the release of the cytosolic enzyme, lactate dehydrogenase (LDH), in the culture medium [38,39]. Briefly, after agmatine treatment, the culture medium and adhering cells recovered after trypsinization were used to measure LDH activity. Membrane integrity was calculated as the ratio of LDH activity in adhering cells versus total LDH activity (the sum of both fractions).

2.3. Determination of intracellular polyamine content and putrescine biosynthesis via ODC

HT-29 cells were cultured with 2 mM agmatine sulfate or 2 mM H_2SO_4 into DMEM containing 3% HS. Twenty-four hours later, the cells were harvested and the cell pellets were