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ORIGINAL ARTICLE

Exploring polyamine regulation by nascent histamine in a human-transfected cell model

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Abstract There are multiple lines of evidence suggesting interplay between histamine and polyamines in several mammalian cell types. However, the complex metabolic context makes it difficult to elucidate the mechanisms involved. Histamine's effects can be elicited after its binding to any of the four subtypes of G-protein coupled histamine membrane receptors. In addition, intracellular histamine can also interfere with polyamine metabolism, since there are several metabolic connections between the synthesis and degradation pathways of both types of amines. In order to dissect the metabolic effects of intracellular histamine on polyamine metabolism, we chose a well-known cell culture line, i.e., the human embryonic kidney 293 cells (HEK-293 cells). Initially, we show that HEK-293 cells lack a polyamine metabolic response to extracellular histamine, even over a wide range of histamine concentrations. HEK-293 cells were transfected with active and inactive versions of human histidine decarboxylase, and changes in many of the overlapping metabolic factors and limiting steps were tested. Overall, the results indicate a regulatory effect of histamine on the post-transcriptional expression of ornithine decarboxylase and suggest that this effect is primarily responsible for the decrease in polyamine synthesis and partial blockade of cell-cycle progression, which should affect cell proliferation rate.

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Abbreviations

BrdU	5′-Bromodeoxyuridine
ECL	Gastric enterochromaffin-like cells
α-FMH	α-Fluoromethylhistidine
HDC	Histidine decarboxylase
HEK-293	Human embryonic kidney 293 cells
HNMT	Histamine *N*-methyltransferase
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
SAM	*S*-Adenosylmethionine
SAMdc	*S*-Adenosylmethionine decarboxylase
SPDS	Spermidine synthase
SPMS	Spermine synthase
SSAT	Spermidine/spermine acetyltransferase

Introduction

Decarboxylation of cationic amino acids produces amines that play important roles in mammalian physiology. Arginine/ornithine-derived polyamines (putrescine, spermidine and spermine) are important for processes related to the cell's life and death. Spermidine and spermine are essential for efficient and high-fidelity macromolecular biosynthesis through direct interaction with nucleic acids (Bachrach 2005b). Polyamine metabolism has been classically considered to be a secondary module of nitrogen metabolism, connected with other metabolic modules mainly through arginine and ornithine (which are necessary for putrescine

formation), methionine and *S*-adenosylmethionine (SAM, necessary for the synthesis of spermidine and spermine) and acetyl-CoA (required for degradation of spermidine and spermine) (Rodriguez-Caso et al. 2006). On the other hand, histamine, the product of histidine decarboxylase (HDC), is mainly related to inflammation (García-Faroldi et al. 2009b; Marone et al. 2003; Thurmond et al. 2008). Nevertheless, it is clear that these two types of biogenic amines share chemical properties and metabolic reactions (Medina et al. 2003, 2005). For instance, histamine degradation involves amino oxidases, acting on the amine itself or its derivative, *N*-methylhistamine, obtained through the action of histamine methyltransferase (a SAM-dependent enzyme). Histaminase, also known as diamine oxidase, is also able to act on putrescine (Agostinelli et al. 2004; Seiler 2004). Tissue transglutaminase or transglutaminase 2 can also use both types of amines as substrates (Griffin et al. 2002; Qiao et al. 2005). Figure 1 shows a schematic of polyamine and histamine metabolic pathways, where the most important regulatory steps and connectors are drawn.

The pathways shown in Fig. 1 coexist in several human cell types involved in different physiological processes, such as the immune response, gastric secretion and neurotransmission (Medina et al. 2003, 2005). Polyamines are essential for human cell survival and the most important elements of their metabolism (ornithine decarboxylase, ODC; *S*-adenosylmethionine decarboxylase, SAMdc and spermidine/spermine acetyltransferase, SSAT) are expressed in most cell types, at least during active proliferation and/or differentiation. On the contrary, histamine is only synthesised by a reduced set of cell types: mast cells and other immune cells, some neurons and gastric enterochromaffin-like (ECL) cells. In mouse mast cells, we observed that extracellular histamine leads to a decrease in intracellular polyamine levels and, in addition, inducers of HDC expression (e.g. TPA and dexamethasone) cause a decrease in both ODC and polyamine levels (Fajardo et al. 2001a, 2001b). However, these effects are at least partially dependent on the complex signal transduction pathways elicited by external stimuli (histamine, phorbol esters and glucocorticoids). It is noteworthy that both sets of amines show antagonistic oscillations during mast cell differentiation (García-Faroldi et al. 2009a) and that histamine-producing cells, even those considered malignant (such as acute cases of mastocytosis, which are considered leukaemias), show a very low proliferation rate compared with other types of leukaemias (Krauth et al. 2006). In gastric

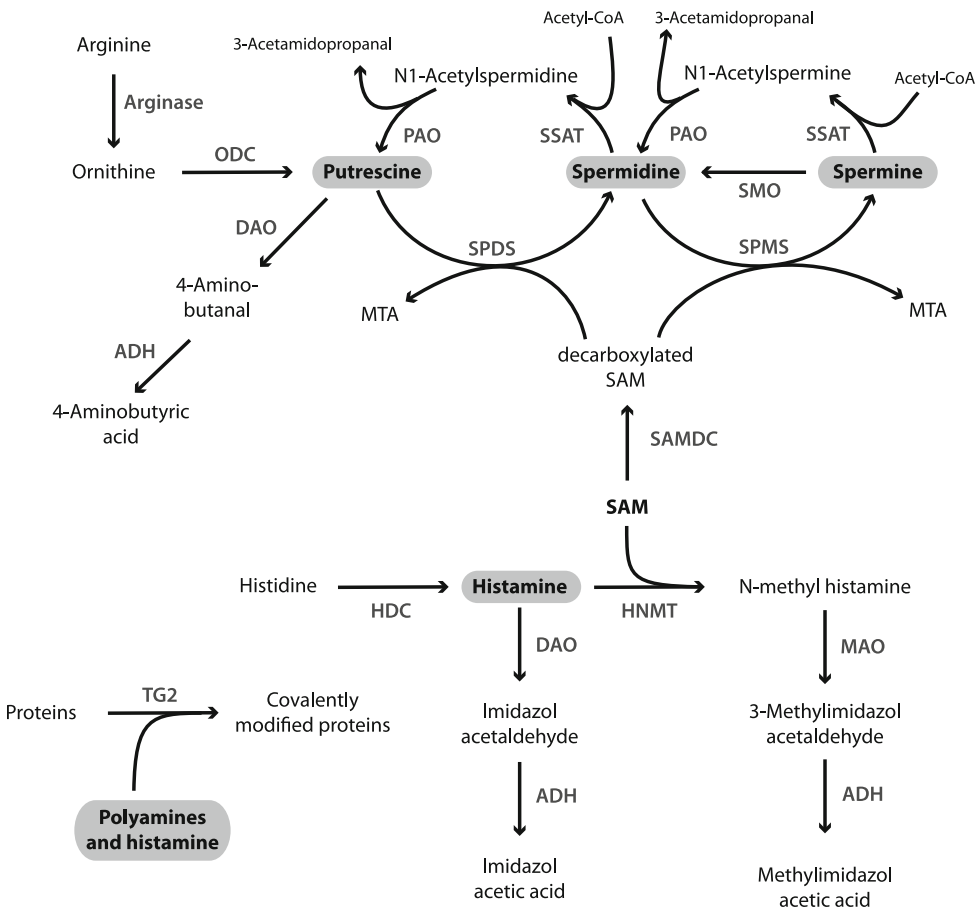

Fig. 1 Interplay between metabolism of polyamines and histamine and the major enzymes involved. *ADH* aldehyde dehydrogenase, *DAO* diamine oxidase, *HDC* histidine decarboxylase, *HNMT* histamine *N*-methyltransferase, *MAO* monoamine oxidase, *MTA* 5′-Methylthioadenosine, *ODC* ornithine decarboxylase, *PAO* polyamine oxidase, *SAM* *S*-adenosylmethionine, *SAMDC* *S*-adenosylmethionine decarboxylase, *SMO* spermine oxidase, *SSAT* spermidine/spermine N^1-acetyltransferase, *SPDS* spermidine synthase, *SPMS* spermine synthase, *TG2* transglutaminase type 2

cancer cells, it has been reported that HDC inhibits the ERK1/2 signal transduction pathway and, consequently, cell proliferation, with no mention to polyamine content (Colucci et al. 2001). However, in this study, the effect was assigned to an interaction between HDC and another proliferative signal, uncharacterised so far.

In order to approach the characterisation of the effects of intracellular histamine on polyamine metabolism in a simpler human model, we chose human embryonic kidney 293 (HEK-293) cells, a proliferating human cell line extensively used for transfection-based experiments. We first confirmed that these cells are unable to express HDC and are insensitive to external histamine at a wide range of concentrations (even above the physiological concentration of the amine). HEK-293 cultures were transfected to transiently express different versions of human HDC, including an active 1-512 version extensively characterised by our group (Engel et al. 1996; Olmo et al. 2000, 2002; Rodríguez-Agudo et al. 2000), an inactive mutant enzyme, and an unprocessed version of the primary translation product partially active in vivo. The results, together with those obtained in the presence or absence of the HDC inhibitor α-fluoromethylhistidine (α-FMH), indicate that polyamine content is a function of the histamine synthesis capacity of the cell. The analysis of the most relevant elements of both metabolic pathways [ODC, SAMdc, spermidine synthase (SPDS), spermine synthase (SPMS), SSAT, polyamine oxidase (PAO) and histamine *N*-methyltransferase (HNMT)] suggests that ODC synthesis is a major sensor of histamine synthesis. This fact, together with the well-known role of ODC and polyamines in cell proliferation, can give insights to explain the inhibitory role of intracellular histamine in many human cell types.

Materials and methods

Cell culture and treatments

Human embryonic kidney 293 cells, purchased from the American Type Culture Collection, were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (Biowhittaker) supplemented with 10% foetal bovine serum (Biowhittaker), 50 IU/ml streptomycin (Biowhittaker), 50 IU/ml penicillin (Biowhittaker) and 1.25 μg/ml amphotericin (Biowhittaker). The human mast cell line HMC-1, generously supplied by Dr. J. Butterfield (Mayo Clinic, Rochester), was cultured at 37°C in a humidified atmosphere containing 5% CO_2 at a starting density of 1×10^5 cells/ml in Iscove's medium (Biowhittaker) supplemented with 10% calf serum (PAA), 2 mM L-glutamine, penicillin (Biowhittaker), streptomycin (Biowhittaker), Fungizone (Gibco), iron supplement (Sigma-Aldrich) and 1.2 mM alpha-thioglycerol (Sigma-Aldrich). Human KU-812-F myelogenous leukaemia lymphoblast cells were supplied by the European Collection of Animal Cell Cultures and maintained in RPMI-1640 medium (Biowhittaker) supplemented with 10% foetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 1.25 μg/ml amphotericin.

Transfections were performed with the FuGENE 6 reagent (Roche Applied Science), according to the manufacturer's recommendations, using 3 μl of FuGENE reagent per μg of DNA. For transfection, cells were seeded on P100 dishes at 2×10^6 cells/ml and transfected cells were analysed 24-h post-transfection. α-FMH (an irreversible inhibitor of HDC, Sigma-Aldrich) was added to transfected cells 1 h prior to transfection. Cells were incubated with histamine (Sigma-Aldrich) at the indicated concentrations for 24 h. For inhibition of amino oxidase activity, cells were transfected in the presence of 1 mM aminoguanidine (Sigma-Aldrich). For experiments on ODC degradation, cells were incubated 24-h post-transfection with 50 μg/ml cycloheximide (Sigma-Aldrich), and ODC activity was measured after 15, 30 and 45 min.

Plasmids

Constructs expressing different versions of human HDC (hHDC) were generated by cloning in frame into the *Bam*HI/*Eco*RI sites of the pCMV-TAG 2B vector (Stratagene). This allowed for hHDC proteins to be tagged at the N-terminus with the FLAG® epitope. hHDC inserts were obtained by reverse transcription-PCR using total RNA from KU-812-F and HMC-1 cells. Constructs were named hHDCX, where X represents the last amino acid of the human HDC protein sequence encoded by the insert. One of the hHDC512 constructs obtained lacked amino acids 262–277 of the hHDC protein sequence, corresponding to the boundary between exons 7 and 8 of the human HDC gene. This construct was named hHDC512Δ262-277. All PCR amplifications were performed using a common sense primer, 5′-AGCCAGAGCGGATCCGAGATGATGGAGC TTGAG-3′, which introduces a *Bam*HI site. The antisense primer used for hHDC512 was 5′-CCTGGCCTGGACT GAATTCTCCTATGCCCCACTGAC-3′. This primer introduces a stop codon after amino acid 512 of the hHDC protein sequence and an *Eco*RI site. For hHDC662, the antisense primer used was 5′-GTTCACAGAATTCC TGAAGTATATCCTC-3′, which introduces an *Eco*RI site after the stop codon of the hHDC cDNA. Reverse transcriptions were performed for 1 h at 42°C in a final volume of 25 μl, containing 5 μg of total RNA (previously denatured for 10 min at 65°C), 1.3 mM dNTPs, 800 nM antisense primer and 200 units of MMLV reverse transcriptase (Promega). After reverse transcription, each PCR was

carried out in a final volume of 50 µl, containing 5 µl of reverse transcription product, 200 µM of each primer, 1.5 mM $MgCl_2$ and three units of *Pfu* DNA polymerase (Stratagene). Thermal cycling was performed in a GeneAmp PCR System 2400 (Perkin Elmer) using the following conditions: denaturing at 95°C for 2 min, 30 cycles of 95°C for 45 s, 60°C for 45 s and 72°C for 45 s and a final extension of 2 min at 72°C.

Western blot analysis

Cell extracts were prepared by adding 1 × SDS-sample buffer (65 mM Tris–HCl containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue) directly to the cell pellets (100 µl per 1 × 10^6 cells) and boiling for 5 min. Samples (20 µl) were subjected to SDS-PAGE on 10% polyacrylamide gels and were subsequently blotted onto nitrocellulose membranes (Pall Corporation), followed by blocking overnight in phosphate-buffered saline (PBS) with 0.1% Tween-20 and 10% non-fat milk at 4°C. All of the following steps were performed at room temperature. Next, membranes were incubated for 1–2 h with primary antibodies against the FLAG epitope (monoclonal anti-FLAG M2-Peroxidase antibody, Sigma-Aldrich) diluted 1:3,500 in PBS/0.1% Tween 20 containing 2% bovine serum albumin. After extensive washing with PBS/0.1% Tween-20, membranes were developed with the SuperSignal West Pico Chemiluminescent Substrate system (Thermo Scientific), according to the manufacturer's instructions. Normalisation of sample loading was performed by re-probing membranes with an anti-beta actin mouse monoclonal antibody (Sigma-Aldrich).

Intra- and extracellular contents of amines and SAM

The intracellular contents of spermidine and spermine were simultaneously determined by fluorimetry after separation of their dansyl derivatives by reverse-phase HPLC, as previously described (García-Faroldi et al. 2009a).

Histamine quantification in cell extracts and cell culture supernatants was performed by ELISA with a kit from DRG (Germany), following the instructions of the manufacturer. Results were normalised to total cell number and are expressed as µmol of histamine per 10^6 cells.

Intracellular contents of SAM and SAH were simultaneously determined using isocratic HPLC with ultraviolet detection at 254 nm, as described elsewhere (She et al. 1994).

Enzymatic activities and degradation and synthesis of ODC

Ornithine decarboxylase and HDC activities were determined by measuring the release of $^{14}CO_2$ from [1-^{14}C]-ornithine and [U-^{14}C]-histidine, as reported previously (Urdiales et al. 1992; Fajardo et al. 2001b). The half-life of ODC was determined by measuring the decline in enzyme activity after adding 50 µg/ml of cycloheximide to the medium 24 h after transfection. Protein synthesis rate was determined as described previously (Urdiales et al. 1992). Briefly, HEK-293 cells were transfected in six-well plates. Twenty-four hours after transfection, cells were washed twice with PBS, and 0.5 ml of culture medium lacking methionine was added. After 5–10 min, culture medium was supplemented with 100 µCi of ^{35}S-methionine (Perkin Elmer, 1,175 Ci/mmol), and the cells were incubated for different times, up to 30 min. The incorporation of radiolabelled methionine into protein was stopped by the addition of an excess of unlabelled methionine. ^{35}S incorporation into the acid-insoluble fraction was determined. For the measurement of relative ODC synthesis, aliquots of supernatant (containing equal amounts of acid-insoluble radioactivity) were incubated with an excess of polyclonal monospecific antibody raised against mouse ODC (kindly provided by Dr. Lisa M. Shantz, Penn State College of Medicine).

The histamine *N*-methyltransferase assay is based on the methylation of histamine by [methyl-^{14}C]S-adenosylmethionine, as described previously by Dr. H. Schwelberger's group (Kufner et al. 2001).

cDNA array and Northern blotting for amine metabolism-related genes

RNA samples were isolated with the GenElute Mammalian Total RNA Kit (Sigma-Aldrich), according to the manufacturer's recommendations. They were spectrophotometrically quantified, and only samples with absorbance ratios (260/280) near 1.9 were used. mRNAs were amplified using MessageAmpII, a RNA amplification kit (Ambion), according to the manufacturer's specifications.

Array hybridisation was performed as described by Chaves et al. (2007) with minor modifications. Briefly, a ^{32}P-labelled target cDNA mixture was synthesised from 1 µg of amplified mRNA and the reagents provided with the transcriptor/1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). The final reaction volume was 50 µl, containing random primers, a mix of dATP, dTTP and dGTP (0.5 nM final concentration) and 50 µCi [α-^{32}P]-dCTP (Perkin Elmer, 3,000 Ci/mmol). Unincorporated nucleotides were removed with Sephadex G50 columns (Sigma-Aldrich). Labelled cDNA was denatured at 95°C before hybridisation.

For Northern blots, 1 µg of amplified mRNA was separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a Nylon membrane and hybridised as described previously (Fajardo et al. 2001a; Urdiales et al. 1992).

Flow cytometry for bromodeoxyuridine incorporation

Incorporation of 5′-bromodeoxyuridine (BrdU) was analysed using the FITC BrdU Flow kit (BD Biosciences), according to the manufacturer's recommendations. For intracellular labelling of FLAG-tagged HDC, a monoclonal anti-FLAG M2-Cy3 antibody (Sigma-Aldrich) was used. Specific fluorescence was detected with a FACSort flow cytometer (BD Biosciences). Cells were gated according to FL2-H (FLAG-Cy3) fluorescence into FLAG-positives (expressing the transfected hHDC clones) and FLAG-negatives (untransfected cells). Cell-cycle distribution of gated cells was calculated using plots of FL3-H (7-AAD) versus FL1-H (BrdU-FITC).

Statistical analysis

Student's unpaired samples *t* test (two tailed) was performed using GraphPad Prism version 5.0b for Mac, GraphPad Software, San Diego, CA, USA, http://www.graphpad.com. Values of $P < 0.05$ were considered to be significant.

Results and discussion

Characterisation of amine-related elements in HEK-293 cells

Human embryonic kidney 293 cells are proliferating cells widely used for transfection experiments. After transfection with pCMV-TAG 2B empty plasmid, HEK-293 cells presented a doubling time of approximately 14 h. This proliferation rate is consistent with moderately high synthesis of polyamines. Polyamine levels are the result of a metabolic bi-cycle (Fig. 1). ODC and SAMdc act as the limiting steps of polyamine synthesis, and SSAT is the main regulatory enzyme for polyamine degradation. It has been demonstrated that this bi-cycle is a very robust system presenting multiple compensatory mechanisms (Rodríguez-Caso et al. 2006). In conditions where SAM (or decarboxylated SAM) is not the limiting step, most of the available putrescine is rapidly converted into spermidine, a polyamine mainly found bound to cytosolic RNA. Spermidine is further converted to spermine, a polyamine described to be mainly located in the nucleus (Igarashi and Kashiwagi 2000). First, we wanted to ensure that all of these polyamine-related enzymes were expressed in our model and that we could detect intracellular polyamine levels. Expression of polyamine metabolism-related gene transcripts was confirmed by a cDNA array previously described by our group (Chaves et al. 2007). This array revealed that proliferating HEK-293 cells (24 h after transfection) express all elements necessary for the polyamine metabolic pathway shown in Fig. 1. The normalised signal values (mean ± standard deviation of two independent experiments) with respect to the mean of GADPH and beta-actin signals were as follows: ODC (52.0 ± 11.0%), SAMdc (27.9 ± 10.4%), SPDS (60.2 ± 3.1%), SPMS (34.7 ± 7.7%), SSAT (13.9 ± 6.0%) and PAO (11.2 ± 3.0%). Intracellular levels of polyamines were also measured in these cells by a previously described HPLC method. Putrescine was not detectable, which is typical of proliferating cells, where the amine is being rapidly converted to spermidine, a polyamine with a more important role in macromolecular synthesis (Bachrach 2005b; Igarashi et al. 1988). The intracellular levels for both detectable polyamines were 4.77 ± 0.39 pmol/μg protein for spermidine and 12.57 ± 1.14 pmol/μg protein for spermine.

On the other hand, we also checked the possibility that this cell line could act either as a histamine-producing or as a histamine-handling cell. Both intracellular histamine level and HDC activity were undetectable in HEK-293 cell cultures. Our previously described cDNA array also contained probes for histamine receptor subtypes H1, H2 and H4. HEK-293 cells seem to express histamine receptors at low levels, with normalised signal values relative to the mean of GADPH and beta-actin signals of 4.9 ± 1.3% for H1R, 8.9 ± 1.1% for H2R and 1.6 ± 0.6% for H4R. Initially, this posed a problem for our experimental strategy, since we wanted to study the effects of intracellular histamine on polyamine metabolism with no interference from other autocrine histamine effects elicited through its binding to cytosolic membrane receptors. Nevertheless, the presence of histamine receptor mRNAs in HEK-293 cells did not prove their functionality or involvement in the regulation of polyamine metabolism. This was checked by treating HEK-293 cells with different extracellular histamine concentrations (from 10 nM to 0.5 mM histamine), covering all ranges of concentrations known to activate histamine receptor responses and even exceeding the highest known physiological concentration of the amine (Thurmond et al. 2008). No major changes were observed on cell number after addition of exogenous histamine. Intracellular polyamine levels were not affected under any of the tested conditions, as shown in Table 1. In C57.1 cells treated with 0.5 mM histamine, we observed an increase in intracellular histamine (Fajardo et al. 2001b), but no histamine accumulation occurred in HEK-293-treated cells. Thus, it seems unlikely that any effect on polyamine metabolism after transient expression of HDC in our model is mediated by histamine receptors and/or intracellular histamine accumulation.

Effects of histamine synthesis on polyamine metabolism

Human embryonic kidney 293 cells were transfected with recombinant plasmids encoding different versions of

Table 1 Intracellular spermidine and spermine concentrations after 24-h treatment with exogenous histamine

Exogenous histamine (μM)	Spermidine (pmol/μg protein)	Spermine (pmol/μg protein)
0	4.95 ± 0.54	17.95 ± 1.34
0.010	5.22 ± 1.10	13.87 ± 3.03
10	5.49 ± 0.78	15.45 ± 2.35
500	4.33 ± 0.14	20.06 ± 0.85

Cells were seeded on P100 dishes at 2×10^6 cells/ml and treated with exogenous histamine for 24 h. Data are mean ± SEM of three independent experiments

human HDC, as described in the "Materials and methods". In the highly homologous rat enzyme, we have demonstrated that a fragment containing amino acids 1–512 is fully active in vitro; its kinetic constants and mechanism of action have been characterised (Moya-García et al. 2005, 2008; Olmo et al. 2002). The primary translation product of the rat enzyme has no activity in vitro. Nevertheless, in transfected cells, residual activity of the rat and human versions can be observed, as also occurred in the present study. The deletion mutant hHDC512Δ262-277 is an inactive version (both in vitro and in vivo) cloned from KU-812-F leukaemia cells and, most probably, was generated by an alternative splicing event at the exon 7–8 boundary. This region codes for an internal alpha-helix close to the catalytic centre, including Asp-273, which is predicted to contact the pyridine ring of PLP and to be essential for enzymatic activity (Moya-García et al. 2008). In fact, other alternative splicing events have been reported during HDC messenger processing in KU-812-F leukaemia cells, leading to generation of an inactive version of the human enzyme (Mamune-Sato et al. 1992).

Histidine decarboxylase protein levels and activities were measured in the four transfected HEK-293 groups 24 h after transfection (Fig. 2a). All hHDC inserts were expressed by transfected cells. The hHDC512 construct was fully active. Lower activity was observed when cells were transfected to express the full-length hHDC662. However, no activity was found in cells transfected with the truncated version hHDC512Δ262-277 or with the empty vector pCMV-TAG 2B (FLAG). In our hands, the full-version hHDC662 and its counterpart of rat are inactive proteins when purified and assayed in vitro. Thus, the activity observed with the full-length version may be due to proteolytic processing (similar to the proteolysis required for maturation of the enzyme) occurring within the cell. In fact, two clear bands were observed after transfection with the hHDC662 plasmid, one corresponding to the primary translation product (apparent molecular weight, 74 kDa) and another one closer to the major 53 kDa band of the

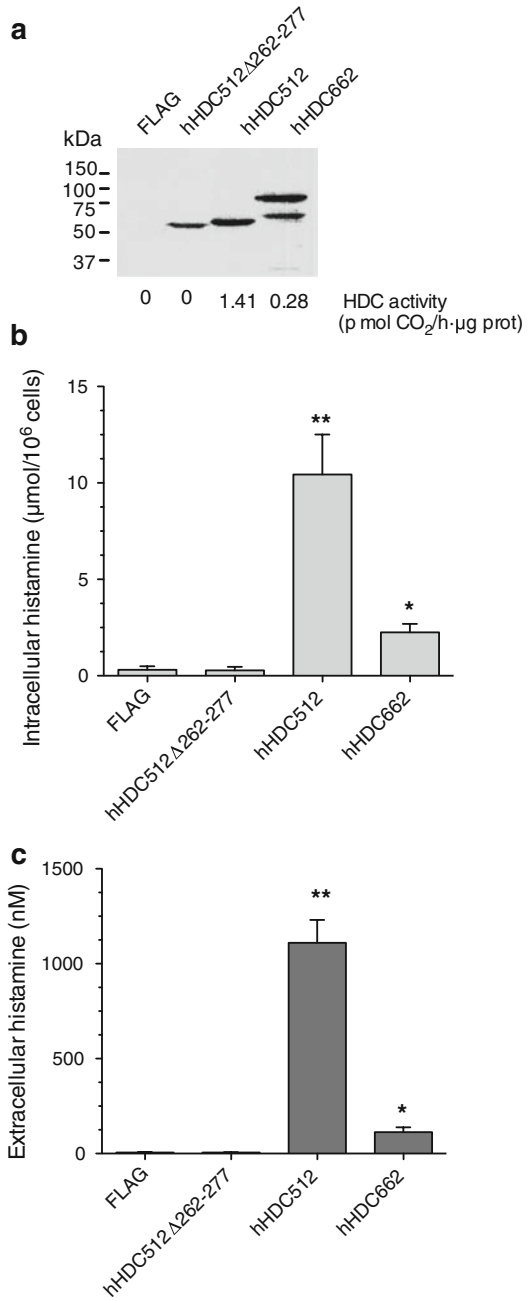

Fig. 2 Expression of hHDC constructs in HEK-293 cells. HEK-293 cells were transfected with pCMV-TAG 2B vector containing different versions of hHDC, as described in "Materials and methods" (as a negative control, pCMV-Tag 2B empty vector, FLAG, was used). Twenty-four hours after transfection, cells were harvested and analysed for HDC protein expression (*panel* **a**) by Western blot with an anti-FLAG M2-peroxidase antibody. HDC activity, expressed as pmol CO_2/h·μg of proteins, of transfected cells is shown below each *lane*. Intracellular (*panel* **b**) and extracellular (*panel* **c**) histamine levels in transfected HEK-293 cells were determined using a histamine assay kit, as described in "Materials and methods." Histamine levels are expressed in nmol per 10^6 cells and represent the mean ± standard error of the mean of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with negative control cells by Student's unpaired sample *t*-test (two-tailed)

mature enzyme (Fig. 2, panel a). Partially active versions of the enzyme have indeed been reported to migrate with apparent molecular weights of 69–54 kDa (Dartsch et al. 1998; Fleming et al. 2004). Twenty-four hours after transfection, we determined the intracellular (Fig. 2b) and extracellular (Fig. 2c) histamine levels, and both correlate with the HDC activities observed for the different constructs used. It is remarkable that more than 99% of the histamine produced by these cells was excreted into the medium, indicating the inability of these cells to store the histamine produced.

We also determined polyamine levels by HPLC after transfection. Putrescine was not detectable under any condition. Spermidine and spermine levels are shown in Fig. 3, panels a and b. It is remarkable that spermidine levels inversely correlate with HDC activity and histamine production capacity in each transfected group. In the cells transfected with hHDC512, spermidine levels were reduced by 73.3 ± 13.9% respect to FLAG-transfected cells. As explained above, even when most of the histamine produced by these cells is released into the medium, interference with spermidine production is unlikely to be due to an autocrine effect of the amine, since the extracellular histamine levels reached by the hHDC512 transfected cells were estimated to be lower than 1.5 μM, and we did not observe any change in the intracellular polyamines after treating the cells with up to 0.5 mM external histamine (see Table 1). Consequently, the decrease in total polyamines, particularly spermidine content, is likely to be caused by either intracellular histamine, called "nascent histamine" (Schneider et al. 2002), or even by an interaction of HDC with other cellular components, as suggested to occur in gastric cancer cells (Colucci et al. 2001). The high inverse correlation degree between HDC activity/intracellular histamine levels and spermidine levels suggests that, most probably, it is the histamine produced by the cells responsible for the observed effect, and not the enzyme's structure, since no effect on spermidine levels was observed upon transfection of inactive versions of HDC (hHDC512Δ262-277 or hHDC662). Nevertheless, we wanted to rule out the possibility that any structural change in these HDC versions was affecting a putative protein–protein interaction required for the inhibitory effect on polyamine levels observed with the active hHDC512 version. For this purpose, we treated transfected HEK-293 expressing hHDC512 with the irreversible inhibitor α-FMH, a substrate analogue, which reacts with HDC and blocks its catalytic action. Figure 4 shows that the catalytic inhibition of the enzyme led to restored spermidine levels, reinforcing the conclusion that the effect on polyamine metabolism is caused by intracellular histamine and not by the HDC protein structure.

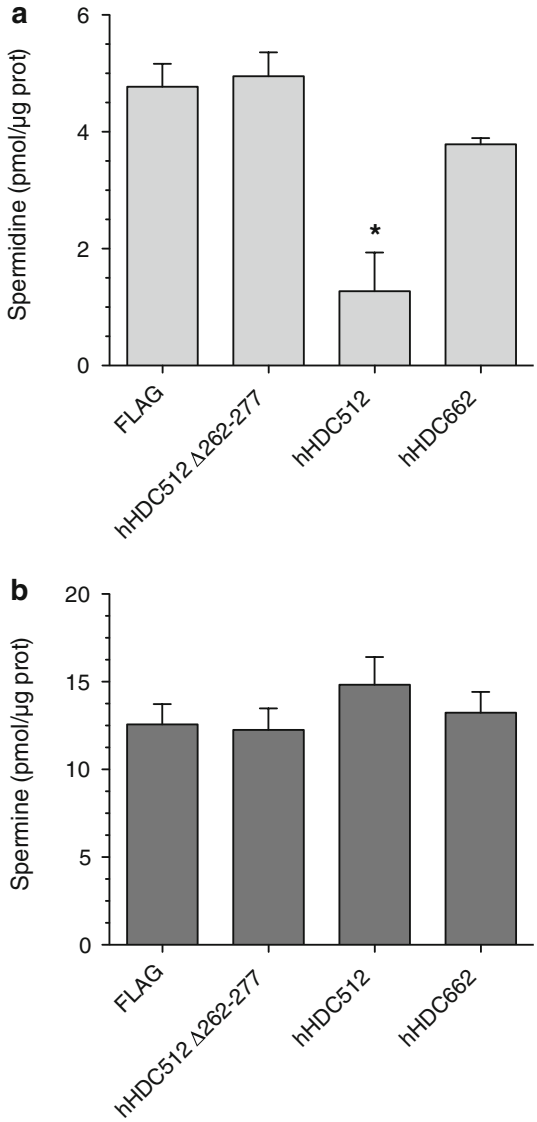

Fig. 3 Intracellular polyamine content in transfected HEK-293 cells. HEK-293 cells were transfected with pCMV-Tag 2B containing different versions of hHDC, as described in "Materials and methods" (as a negative control, pCMV-Tag 2B empty vector, FLAG, was used). Twenty-four hours after transfection, cells were harvested and analysed for intracellular levels of spermidine (*panel* **a**) and spermine (*panel* **b**) by HPLC. Results are expressed as pmol of each polyamine per μg of protein and represent mean ± SEM of three independent experiments. $*P < 0.05$ compared with negative control cells by Student's unpaired sample *t*-test (two-tailed)

Exploring the mechanism responsible for the inhibitory effect of intracellular histamine on spermidine synthesis

As mentioned above, polyamine metabolism exhibits a very complex regulation mechanism, where disturbances at any point can be silenced or transmitted to other elements in the bi-cycle. Thus, we wanted to consider different possibilities to explain the effect of histamine on different

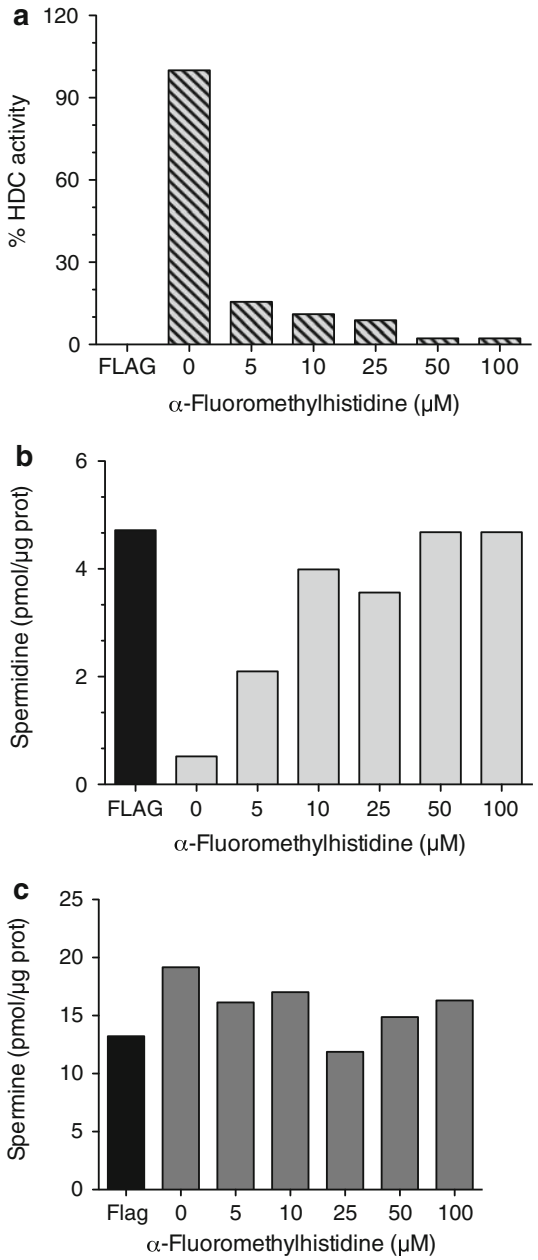

Fig. 4 Effect of α-FMH on HDC activity and intracellular content of polyamines in transfected HEK293 cells. HEK-293 cells were transfected with pCMV-Tag 2B containing hHDC512, as described in "Materials and methods" (as a negative control, pCMV-Tag 2B empty vector, FLAG, was used). One hour before transfection, the indicated concentration of α-FMH was added to the culture medium, and 24 h after transfection, HDC activity (*panel* **a**), intracellular spermidine (*panel* **b**) and spermine (*panel* **c**) concentration were determined. HDC activity was normalised to the activity in cells transfected with hHDC512 in the absence of α-FMH. Results shown are from a representative experiment

elements of the pathway. One of the most intuitive hypotheses to explain how histamine synthesis could affect steady-state spermidine levels is to propose that it diminishes spermidine synthase activity or the availability of SAM or decarboxylated SAM (the donor of the aminopropyl-group added to putrescine). In fact, reduced SAM levels would not be surprising, since intracellular degradation of histamine could involve methylation by HNMT, a SAM-dependent enzyme. However, we could not get any evidence supporting any direct effect on the mentioned possibilities. We could not detect HNMT activity in HEK-293 cells, nor any significant change in SAM levels (results not shown), even though SAM levels have been described as one of the most important limiting factors in polyamine biosynthesis (Rodríguez-Caso et al. 2006). Moreover, no significant differences could be detected in our expression array concerning spermidine synthase or SAMdc transcripts. In addition, any effect on decarboxylated SAM availability should have been reflected in spermine levels also, since its synthesis consumes a decarboxylated SAM molecule. However, spermine levels remained almost unchanged in our experiments, perhaps due to the fact that most of this amine is kept bound to DNA, with little interchange with the cytosolic polyamine pool, as described by other authors in the field (Igarashi and Kashiwagi 2000).

Since the decrease in spermidine was determined not to be due to a reduced availability of one of its precursors (SAM), we checked the possibility that a reduced availability of the other precursor, putrescine, could account for the decreased spermidine levels observed upon expression of hHDC512. Putrescine could not be detected under any condition, thus suggesting that either it is rapidly consumed by degradation systems (e.g. diamine oxidase/histaminase) (Seiler 2004) or rapidly converted to spermidine, thereby acting as a limiting factor in spermidine synthesis. To evaluate if an augmented rate of putrescine degradation by amino oxidases (which might be induced by the increased levels of histamine acquired after hHDC512 expression) could justify the reduced spermidine levels observed in cells expressing hHDC512, we treated these cells with 1 mM aminoguanidine (a well-known diamine oxidase inhibitor) and determined polyamine levels. As shown in Fig. 5, the presence of the histaminase inhibitor does not alter the polyamine profile. These results, therefore, rule out an important contribution of part of the degradative pathways in our model.

Taken together, the observed results pointed to a limiting role of putrescine in spermidine synthesis due to a reduced synthesis of this diamine even under control conditions, which could be exacerbated in the presence of intracellular histamine. The activity of ODC, the enzyme responsible for putrescine synthesis, is indeed the limiting step in polyamine biosynthesis in most cases (Hayashi 1989; Russel 1989; van Daalen Wetters et al. 1989) as well as a very good early sensor of any anti-proliferative stimuli (Urdiales et al. 1996). As shown in Fig. 6a, ODC activity

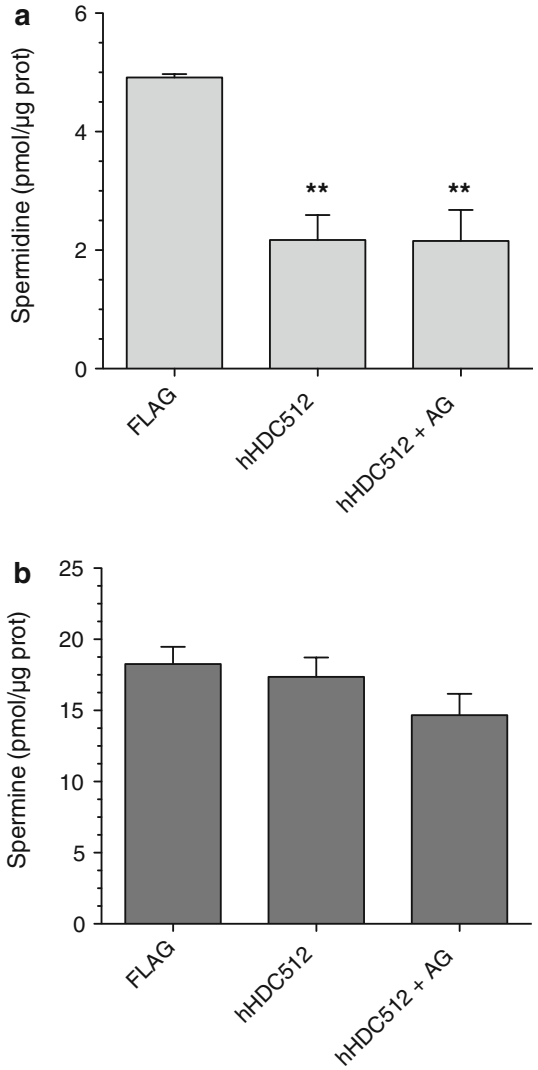

Fig. 5 Effect of aminoguanidine on intracellular polyamine content in transfected HEK-293 cells. HEK-293 cells were transfected with pCMV-Tag 2B containing hHDC512 in the absence or presence 1 mM aminoguanidine (as a control, pCMV-Tag 2B empty vector, FLAG, was used in the absence of aminoguanidine). Twenty-four hours after transfection, spermidine (*panel* **a**) and spermine (*panel* **b**) levels were determined by HPLC. Results are expressed as pmol of each polyamine per µg protein and represent mean ± SEM of three independent experiments. $^{**}P < 0.01$ compared with control cells by Student's unpaired sample *t*-test (two-tailed)

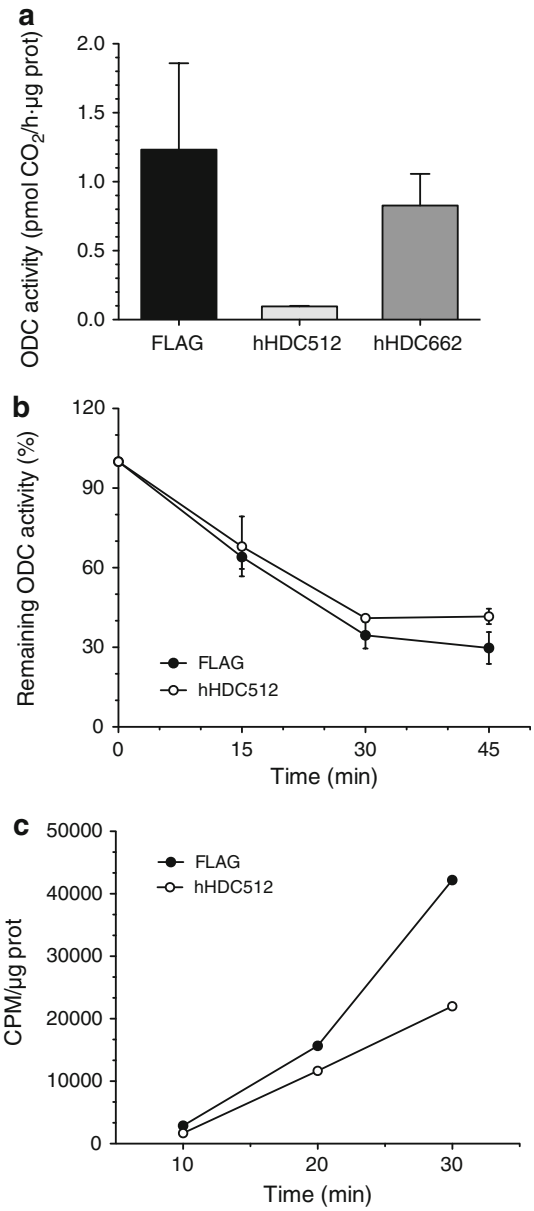

Fig. 6 Ornithine decarboxylase activity, half-life and ^{35}S-methionine incorporation in transfected HEK-293 cells. Twenty-four hours after transfection, cells were harvested and ODC activity was analysed (*panel* **a**). ODC activity is expressed as pmol CO_2/h·µg protein and represents the mean ± SEM of three independent experiments. For determination of ODC half-life (*panel* **b**), 50 µg/ml cycloheximide was added to the culture medium 24 h after transfection, and ODC activity was determined at the indicated times for cells transfected with empty pCMV-TAG 2B vector (FLAG, *filled circles*) or with hHDC512 plasmid (*open circles*). ODC activity was normalised to the activity at time zero; results are mean ± SEM of three independent experiments. Protein synthesis rate was determined (*panel* **c**) in control (FLAG, *filled circles*) and hHDC512-transfected cells (*open circles*). See "Materials and methods" for details

was dramatically reduced (by more than 90%) when the fully active hHDC512 construct was expressed. This effect was neither attributable to reduced levels of ODC mRNA, as evidenced by Northern blot experiments (results not shown), nor assignable to an alteration in ODC degradation rate, as deduced by the estimated ODC half-life values that were very similar for both controls and hHDC512-transfected cells (22.43 vs. 25.02 min respectively; Fig. 6b).

Protein steady-state levels are defined by synthesis and degradation rates. Since the rate of ODC degradation seems to be unaltered by the augmented levels of histamine, we also investigated the rate of protein synthesis by measuring ^{35}S-labelled methionine incorporation. We observed that

the expression of hHDC512 protein inhibits total protein synthesis rate by 38% (Fig. 6c). Given that ODC exhibits a rapid turnover rate (as shown above), changes (specific or not) in protein synthesis can rapidly regulate ODC protein levels within the cell. We thus also attempted to estimate specific ^{35}S incorporation into ODC by immunoprecipitation, as reported previously (Urdiales et al. 1992). However, under our experimental conditions (very low ODC levels and a limited amount of antibody against ODC), a specific ODC band could not be clearly detected or quantified, even in control cells.

Polyamines are essential for the growth and function of normal cells (Bachrach 2004; Montañez et al. 2007). It has been known for many years that ODC levels change during the cell cycle (Heby 1981; Thomas and Thomas 2001), and that polyamines are modifiers of cell-cycle kinetics

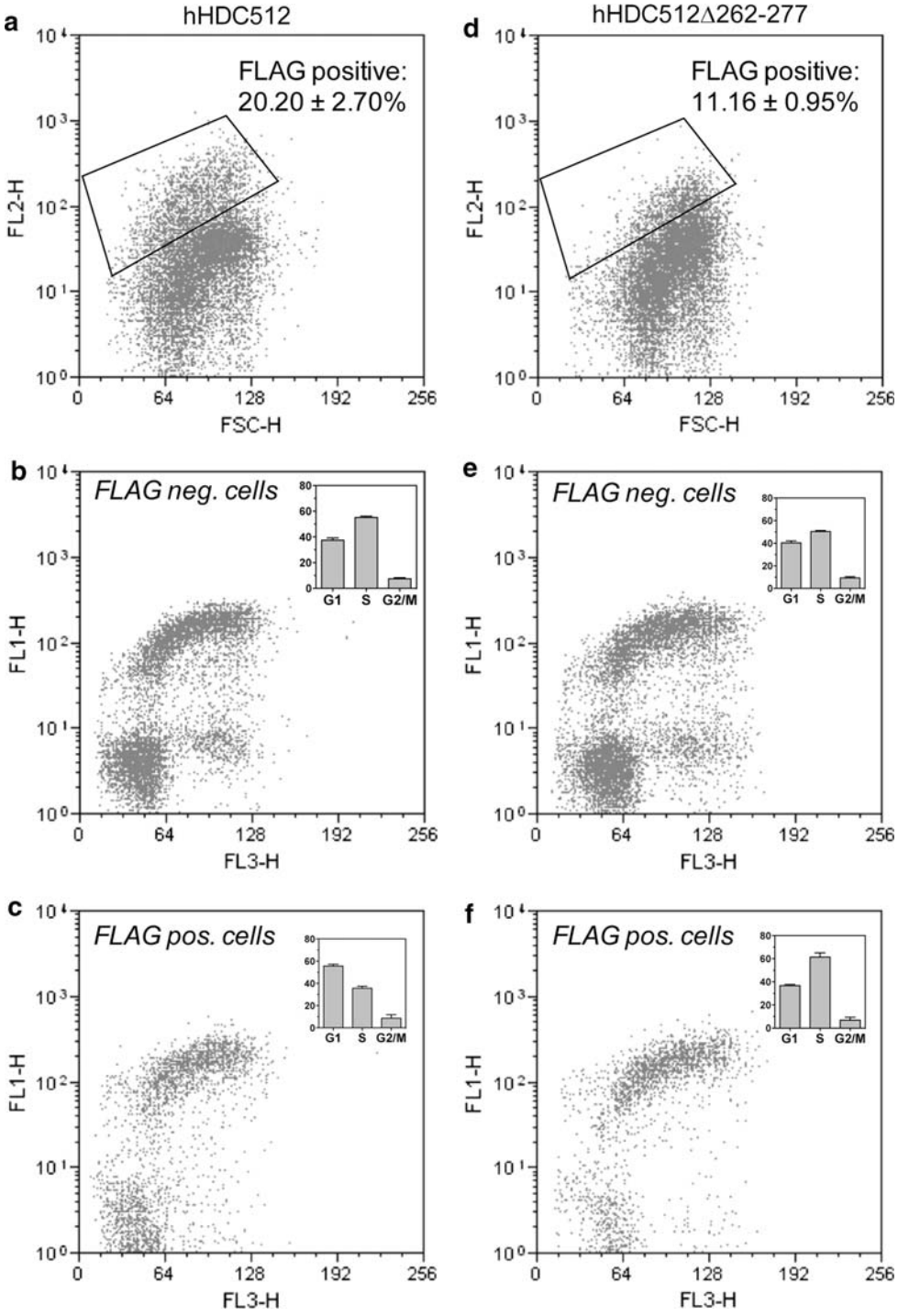

Fig. 7 Bromodeoxyuridine incorporation in transfected HEK-293 cells. HEK-293 cells were transfected with active hHDC512 (*panels* **a**–**c**) or the inactive form hHDC512Δ262-277 (*panels* **d**–**f**). BrdU was added for 45 min to label the cells 24 h after transfection. BrdU incorporation (FL1-H), DNA content (FL3-H) and transfection status of the cells (FL2-H) were analysed by flow cytometry using a FITC BrdU Flow Kit and an anti-FLAG M2-Cy3 antibody to label transfected cells (FLAG-positive), as described in "Materials and methods." *Panels* **a** and **d** show percentages of transfected cells (FL2-positive cells). Results represent mean ± SEM of two independent experiments. *Panels* **b** and **e** show cell-cycle distribution of FLAG-negative cells. *Panels* **c** and **f** show cell-cycle distribution of FLAG-positive cells. Insets in *panels* **b**, **c**, **e** and **f** show cell-cycle distribution of gated cells, with results representing mean ± SEM of two independent experiments

(Koomoa et al. 2008; Kramer et al. 2001; Nasizadeh et al. 2005; Oredsson 2003; Ray et al. 1999). Consequently, we wanted to check if cell proliferation was affected in our model upon histamine synthesis. HEK-293 cells were transfected with different versions of hHDC, and cell-cycle distribution was analysed by flow cytometry using BrdU incorporation, with transfected cells identified using a monoclonal anti-FLAG M2-Cy3 antibody. Cells were gated according to FL2-H (FLAG-Cy3) fluorescence into FLAG-positives (expressing hHDC versions) and FLAG-negatives (not expressing hHDC versions). As shown in Fig. 7a, 20.20 ± 2.70% of the cells were FLAG-positive when the active enzyme hHDC512 was used. However, when the inactive hHDC512Δ262-277 construct was used, only 11.16 ± 0.95% of the cells were FLAG-positive. Cell-cycle distribution of gated cells was calculated using FL3-H (7-AAD) versus FL-1 (BrdU-FITC) plots. No differences were found in the cell-cycle distribution of FLAG-negative cells with either hHDC construct (Fig. 7b, e) or between FLAG-negative cells and cells transfected with empty pCMV-TAG 2B (results not shown). However, BrdU incorporation was inhibited in FLAG-positive cells transfected with the active construct hHDC512 (35.72 ± 1.72% of cells in S-phase, Fig. 7c), compared with cells transfected with inactive hHDC512Δ262-277 (61.43 ± 3.62% of cells in S-phase, Fig. 7f). On the other hand, transfection with the active form of hHDC induced an accumulation of cells in G1 phase (55.63 ± 1.52%, Fig. 7c). These results agree with previously reported data indicating that depletion of polyamines results in a cell-growth arrest; e.g., treatment with α-difluoromethylornithine, an inhibitor of ODC, results in a G1-phase block (Koomoa et al. 2008; Kramer et al. 2001; Oredsson 2003; Ray et al. 1999).

Concluding remarks

Only a very limited set of mammalian cells is able to synthesise histamine. Several of these cells (e.g. mast cells, neurons and ECL cells) accumulate histamine in specialised granules, and others, such as some immune cells, only produce histamine in advanced stages of differentiation (Medina et al. 2003). On the contrary, polyamines are essential for cell proliferation (Bachrach 2004; Montañez et al. 2007). Evidence has accumulated pointing to an antagonistic relationship between polyamine and histamine metabolism in several mammalian cell models, as mentioned in "Introduction". However, it is difficult to distinguish between effects caused by intracellular histamine and those elicited by the amine through its membrane receptors. In this paper, we dissected the effects of intracellular histamine on polyamine metabolism, i.e., what several authors have termed the effect of "nascent histamine" (Schneider et al. 2002). Protein synthesis, and consequently ODC synthesis, seems to be a major target of the inhibitory effects of "nascent histamine." Consequently, spermidine levels decrease, and cell-cycle progression is partially blocked. At this point, it is not easy to discern the first target of this effect. It is possible that histamine initially alters an unidentified proliferative signalling element, resulting in a general reduction in protein synthesis. A reduced protein synthesis rate, together with rapid ODC turnover, could explain the dramatic decrease in enzyme activity observed. Nevertheless, it is clear that a reduction in ODC synthesis itself can exert a positive feedback, amplifying an antiproliferative signal. It is well documented that reduced spermidine levels (caused, for instance, by an ODC inhibitor) affect protein synthesis machinery and rate. In fact, spermidine is essential for the formation of proper RNA conformation and synthesis of hypusine (a component of eIF-5A) (Hyvonen et al. 2007; Igarashi and Kashiwagi 2000; Wolff et al. 2007). It is also well known that ODC is one of the early sensors of antiproliferative stimuli (Bachrach 2005a) and that polyamine deprivation blocks cell-cycle progression (Koomoa et al. 2008; Kramer et al. 2001; Oredsson 2003; Ray et al. 1999). Thus, this work implicates ODC and polyamine metabolism as some of the relevant targets of the antiproliferative effects of "nascent histamine." Our results can contribute to the understanding of the physiology of histamine-producing cells and their associated pathologies.

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References

Agostinelli E, Arancia G, Vedova LD, Belli F, Marra M, Salvi M, Toninello A (2004) The biological functions of polyamine oxidation products by amine oxidases: perspectives of clinical applications. Amino Acids 27:347–358

Bachrach U (2004) Polyamines and cancer: minireview article. Amino Acids 26:307–309

Bachrach U (2005a) Immunohistochemical detection of ornithine decarboxylase as a measure of chemosensitivity testing. Methods Mol Med 110:197–211

Bachrach U (2005b) Naturally occurring polyamines: interaction with macromolecules. Curr Protein Pept Sci 6:559–566

Chaves P, Correa-Fiz F, Melgarejo E, Urdiales JL, Medina MA, Sánchez-Jiménez F (2007) Development of an expression macroarray for amine metabolism-related genes. Amino Acids 33:315–322

Colucci R, Fleming JV, Xavier R, Wang TC (2001) L-Histidine decarboxylase decreases its own transcription through downregulation of ERK activity. Am J Physiol Gastrointest Liver Physiol 281:G1081–G1091

Dartsch C, Chen D, Persson L (1998) Multiple forms of rat stomach histidine decarboxylase may reflect posttranslational activation of the enzyme. Regul Pept 77:33–41

Engel N, Olmo MT, Coleman CS, Medina MA, Pegg AE, Sánchez-Jiménez F (1996) Experimental evidence for structure-activity features in common between mammalian histidine decarboxylase and ornithine decarboxylase. Biochem J 320(Pt 2):365–368

Fajardo I, Urdiales JL, Medina MA, Sánchez-Jiménez F (2001a) Effects of phorbol ester and dexamethasone treatment on histidine decarboxylase and ornithine decarboxylase in basophilic cells. Biochem Pharmacol 61:1101–1106

Fajardo I, Urdiales JL, Paz JC, Chavarría T, Sánchez-Jiménez F, Medina MA (2001b) Histamine prevents polyamine accumulation in mouse C57.1 mast cell cultures. Eur J Biochem 268:768–773

Fleming JV, Fajardo I, Langlois MR, Sánchez-Jiménez F, Wang TC (2004) The C-terminus of rat L-histidine decarboxylase specifically inhibits enzymic activity and disrupts pyridoxal phosphate-dependent interactions with L-histidine substrate analogues. Biochem J 381:769–778

García-Faroldi G, Correa-Fiz F, Abrighach H, Berdasco M, Fraga MF, Esteller M, Urdiales JL, Sánchez-Jiménez F, Fajardo I (2009a) Polyamines affect histamine synthesis during early stages of IL-3-induced bone marrow cell differentiation. J Cell Biochem. doi:10.1002/jcb.22246

García-Faroldi G, Sánchez-Jiménez F, Fajardo I (2009b) The polyamine and histamine metabolic interplay in cancer and chronic inflammation. Curr Opin Clin Nutr Metab Care 12:59–65

Griffin M, Casadio R, Bergamini CM (2002) Transglutaminases: nature's biological glues. Biochem J 368:377–396

Hayashi S (1989) Multiple mechanism for the regulation of mammalian ornithine decarboxylase. In: Hayashi H (ed) Ornithine decarboxylase: biology, enzymology, and molecular biology. Pergamon Press, New York, pp 35–46

Heby O (1981) Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19:1–20

Hyvonen MT, Keinanen TA, Cerrada-Gimenez M, Sinervirta R, Grigorenko N, Khomutov AR, Vepsalainen J, Alhonen L, Janne J (2007) Role of hypusinated eukaryotic translation initiation factor 5A in polyamine depletion-induced cytostasis. J Biol Chem 282:34700–34706

Igarashi K, Kashiwagi K (2000) Polyamines: mysterious modulators of cellular functions. Biochem Biophys Res Commun 271:559–564

Igarashi K, Ito K, Sakai Y, Ogasawara T, Kashiwagi K (1988) Regulation of protein synthesis by polyamines. Adv Exp Med Biol 250:315–330

Koomoa DL, Yco LP, Borsics T, Wallick CJ, Bachmann AS (2008) Ornithine decarboxylase inhibition by alpha-difluoromethylornithine activates opposing signaling pathways via phosphorylation of both Akt/protein kinase B and p27Kip1 in neuroblastoma. Cancer Res 68:9825–9831

Kramer DL, Chang BD, Chen Y, Diegelman P, Alm K, Black AR, Roninson IB, Porter CW (2001) Polyamine depletion in human melanoma cells leads to G1 arrest associated with induction of p21WAF1/CIP1/SDI1, changes in the expression of p21-regulated genes, and a senescence-like phenotype. Cancer Res 61:7754–7762

Krauth MT, Agis H, Aichberger KJ, Simonitsch-Klupp I, Mullauer L, Mayerhofer M, Bohm A, Horny HP, Valent P (2006) Immunohistochemical detection of histidine decarboxylase in neoplastic mast cells in patients with systemic mastocytosis. Hum Pathol 37:439–447

Kufner MA, Ulrich P, Raithel M, Schwelberger HG (2001) Determination of histamine degradation capacity in extremely small human colon samples. Inflamm Res 50(Suppl. 2):S96–S97

Mamune-Sato R, Yamauchi K, Tanno Y, Ohkawara Y, Ohtsu H, Katayose D, Maeyama K, Watanabe T, Shibahara S, Takishima T (1992) Functional analysis of alternatively spliced transcripts of the human histidine decarboxylase gene and its expression in human tissues and basophilic leukemia cells. Eur J Biochem 209:533–539

Marone G, Granata F, Spadaro G, Genovese A, Triggiani M (2003) The histamine-cytokine network in allergic inflammation. J Allergy Clin Immunol 112:S83–S88

Medina MA, Urdiales JL, Rodríguez-Caso C, Ramírez FJ, Sánchez-Jiménez F (2003) Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications. Crit Rev Biochem Mol Biol 38:23–59

Medina MA, Correa-Fiz F, Rodríguez-Caso C, Sánchez-Jiménez F (2005) A comprehensive view of polyamine and histamine metabolism to the light of new technologies. J Cell Mol Med 9:854–864

Montañez R, Sánchez-Jiménez F, Aldana-Montes JF, Medina MA (2007) Polyamines: metabolism to systems biology and beyond. Amino Acids 33:283–289

Moya-García AA, Medina MA, Sánchez-Jiménez F (2005) Mammalian histidine decarboxylase: from structure to function. Bioessays 27:57–63

Moya-García AA, Ruiz-Pernía J, Martí S, Sánchez-Jiménez F, Tuñón I (2008) Analysis of the decarboxylation step in mammalian histidine decarboxylase. A computational study. J Biol Chem 283:12393–12401

Nasizadeh S, Myhre L, Thiman L, Alm K, Oredsson S, Persson L (2005) Importance of polyamines in cell cycle kinetics as studied in a transgenic system. Exp Cell Res 308:254–264

Olmo MT, Urdiales JL, Pegg AE, Medina MA, Sánchez-Jiménez F (2000) In vitro study of proteolytic degradation of rat histidine decarboxylase. Eur J Biochem 267:1527–1531

Olmo MT, Sánchez-Jiménez F, Medina MA, Hayashi H (2002) Spectroscopic analysis of recombinant rat histidine decarboxylase. J Biochem 132:433–439

Oredsson SM (2003) Polyamine dependence of normal cell-cycle progression. Biochem Soc Trans 31:366–370

Qiao SW, Piper J, Haraldsen G, Oynebraten I, Fleckenstein B, Molberg O, Khosla C, Sollid LM (2005) Tissue transglutaminase-mediated formation and cleavage of histamine-gliadin complexes: biological effects and implications for celiac disease. J Immunol 174:1657–1663

Ray RM, Zimmerman BJ, McCormack SA, Patel TB, Johnson LR (1999) Polyamine depletion arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. Am J Physiol 276:C684–C691

Rodríguez-Agudo D, Olmo MT, Sánchez-Jiménez F, Medina MA (2000) Rat histidine decarboxylase is a substrate for m-calpain in vitro. Biochem Biophys Res Commun 271:777–781

Rodríguez-Caso C, Montañez R, Cascante M, Sánchez-Jiménez F, Medina MA (2006) Mathematical modeling of polyamine metabolism in mammals. J Biol Chem 281:21799–21812

Russel DH (1989) The physiological significance of ornithine decarboxylase. In: Bachrach U, Heimer YM (eds) The physiology of polyamines. I. CRC, Boca Raton, pp 255–280

Schneider E, Rolli-Derkinderen M, Arock M, Dy M (2002) Trends in histamine research: new functions during immune responses and hematopoiesis. Trends Immunol 23:255–263

Seiler N (2004) Catabolism of polyamines. Amino Acids 26:217–233

She QB, Nagao I, Hayakawa T, Tsuge H (1994) A simple HPLC method for the determination of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rat tissues: the effect of vitamin B6

deficiency on these concentrations in rat liver. Biochem Biophys Res Commun 205:1748–1754

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244–258

Thurmond RL, Gelfand EW, Dunford PJ (2008) The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. Nat Rev Drug Discov 7:41–53

Urdiales JL, Matés JM, Núñez de Castro I, Sánchez-Jiménez FM (1992) Chlorpheniramine inhibits the ornithine decarboxylase induction of Ehrlich carcinoma growing in vivo. FEBS Lett 305:260–264

Urdiales JL, Morata P, Núñez de Castro I, Sánchez-Jiménez F (1996) Antiproliferative effect of dehydrodidemnin B (DDB), a depsipeptide isolated from Mediterranean tunicates. Cancer Lett 102:31–37

van Daalen Wetters T, Brabant M, Coffino P (1989) Regulation of mouse ornithine decarboxylase activity by cell growth, serum and tetradecanoyl phorbol acetate is governed primarily by sequences within the coding region of the gene. Nucleic Acids Res 17:9843–9860

Wolff EC, Kang KR, Kim YS, Park MH (2007) Posttranslational synthesis of hypusine: evolutionary progression and specificity of the hypusine modification. Amino Acids 33:341–350