Histamine inhibits adrenocortical cell proliferation but does not affect steroidogenesis

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Abstract

Histamine (HA) is a neurotransmitter synthesized in most mammalian tissues exclusively by histidine decarboxylase enzyme. Among the plethora of actions mediated by HA, the modulatory effects on steroidogenesis and proliferation in Leydig cells (LCs) have been described recently. To determine whether the effects on LCs reported could be extrapolated to all steroidogenic systems, in this study, we assessed the effect of this amine on adrenal proliferation and steroidogenesis, using two adrenocortical cell lines as experimental models, murine Y1 cells and human NCI-H295R cells. Even when steroidogenesis was not modified by HA in adrenocortical cells, the biogenic amine inhibited the proliferation of H295R cells. This action was mediated by the activation of HRH1 subtype and an increase in the production of inositol phosphates as second messengers, causing cell-cycle arrest in the G2/M phase. These results indicate a new role for HA in the proliferation of human adrenocortical cells that could contribute to a better understanding of tumor pathology as well as to the development of new therapeutic agents.

Key Words

- histamine
- adrenocortical cells
- proliferation
- steroidogenesis

Introduction

Histamine (HA) is a monoamine neurotransmitter synthesized exclusively by histidine decarboxylase (HDC) in most mammalian tissues. It functions through the activation of four different receptor subtypes, namely HRH1, HRH2, HRH3, and HRH4, with all of them being members of the G-protein-coupled receptor family and associated with different signal transduction pathways (Jones & Kearns 2010).

HA has been shown to stimulate steroidogenesis in testicular parenchyma of the golden hamster (Mayerhofer et al. 1989). Our group extended those observations reporting a dual concentration-dependent effect of the amine on steroidogenesis in MA-10 murine Leydig cells (LCs) and in purified rat LCs (Mondillo et al. 2005). These results revealed a novel biological activity of HA, namely the negative modulation of testicular steroid synthesis via HRH1. In addition, our results also indicated that nitric oxide synthase (NOS) activation is the main intracellular mechanism by which HA exerts its anti-steroidogenic effects (Mondillo et al. 2009).

Over the last few years, the proliferative actions of HA have become the subject of increasing interest as is
evidenced by the increasing number of scientific publications (Falus et al. 2011). In this respect, it has been shown that HA can act as both an anti-mitogenic (Criaco et al. 2006, Petit-Bertron et al. 2009, Meng et al. 2011) and a mitogenic (Molina-Hernandez & Velasco 2008, Francis et al. 2009, Medina et al. 2011, Stoyanov et al. 2012) agent, depending on the cell type and the HA receptor pattern expressed. In particular our recent studies have shown, for the first time to our knowledge, the proliferative effect of HA in MA-10 LCs, mediated via HRH2 activation and increased cAMP production and ERK phosphorylation (Pagotto et al. 2012).

Among steroidogenic tissues, the adrenal cortex is responsible for the production of steroid hormones essential for life. It has been demonstrated that HA is able to regulate adrenal steroidogenesis in rats and dogs by acting on the CNS via HRH1 through an adrenocorticotropic hormone (ACTH)-independent mechanism (Bugajski 1984, Tsujimoto et al. 1993). Furthermore, it is known that the chromaffin cells of the adrenal medulla are capable of responding to HA via HRH1, stimulating the secretion of catecholamines and neuropeptides, which, in turn, act in a paracrine way on cortical cells regulating adrenal cortisol secretion (Bunn & Boyd 1992). The presence of HA in the adrenal gland has been demonstrated in guinea pigs and rats, with most of it being present in the cortex (Endo & Ogura 1974). Possible sources of cortical HA would be the subpopulations of adult chromaffin cells present in the medulla (Tuominen et al. 1993), the endings of the splanchnic nerve, and the mast cells that are arranged surrounding adrenal arterioles, near the capsule (Hinson et al. 1989, Borges 1994). This background supports an indirect effect of HA on the regulation of adrenal steroidogenesis. However, the literature concerning a possible direct effect of the amine on adrenocortical cells is controversial, in part because it comes from studies on different species and those utilizing experimental approaches in which adrenocortical cells are partially or even not isolated. For example, studies with perfused dog adrenal glands or guinea pig primary cultures have referred to a direct effect of HA on cortisol secretion (Matsumoto et al. 1981, Aikawa et al. 1986), while others have postulated the direct action of this amine only on chromaffin cells, using a bovine model (Orso et al. 1997, Yoshida et al. 1997). To date, no studies that have included the analysis of a possible direct action of HA on pure adrenocortical cell lines, which would define the situation unequivocally, have been reported.

Szabó et al. (2009) have recently published a report that HDC expression and HA content are highest in normal tissues, lower in benign tumors, and significantly lower in adrenocortical carcinoma (ACC).

Considering the information given above and our previous findings about the ability of HA to regulate testicular steroidogenesis, the aim of the present study was to assess the direct effect of this amine on adrenal steroidogenesis and proliferation. To achieve this goal, we used two well-characterized adrenocortical cell lines, human NCI-H295R and murine Y1, which serve as established models for studies of adrenal cortical neoplasia and human adrenal steroidogenesis (Gazdar et al. 1990, Rodriguez et al. 1997, Rainey et al. 2004).

Materials and methods

Materials

Histamine dihydrochloride, HRH1 agonist 2-((3-trifluoromethyl)phenyl)histamine dimaleate (FMPH), HRH1 antagonist pyrilamine, HRH2 agonist amthamine (AMTH), HRH3 agonist imetit (IMET), HRH4 agonist VUF 8430 (VUF), TME-cAMP, BSA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), transferrin, selenium, glutamine and NaHCO₃, phospholipase C inhibitor (U-73122), PLC inactive analog inhibitor (U-73343), doxorubicin, epigallocatechin gallate (EGCG), and mouse monoclonal anti-β-tubulin and caspase-3 antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). [³H]-Pyrilamine, Na[¹²⁵]I, [³H]-thymidine, and myo-[³H]-inositol were purchased from NEN (Boston, MA, USA). Cell culture supplies were obtained from Gibco-BRL. Dowex AG-I-X8 resin was procured from Bio-Rad (Hercules, CA, USA). TME-cAMP was radiolabeled with Na[¹²⁵]I in our laboratory by the chloramine-T method (specific action 600 Ci/mmol). Antibody for cAMP was provided by Dr A F Parlow (NHPP). Specific antibodies for progesterone and STAR were gifts from Dr Bussmann (IBYME-CONICET-Argentina) and Dr Miller (University of California, San Francisco) respectively. Anti-HA antibody was obtained from Alpha Diagnostic (San Antonio, TX, USA). Rabbit anti-HDC antibody was obtained from Santa Cruz Biotechnology. Secondary conjugated anti-rabbit antibody coupled to peroxidase was purchased from Vector Labs (Burlingame, CA, USA). DMEM/F12 medium (Gibco) and HyClone supplemented calf serum (Thermo Scientific; Waltham, MA, USA) were obtained from Invitrogen. Insulin was a gift from Laboratorios Beta (Buenos Aires, Argentina). Other reagents used were of the best grade available and were obtained from common suppliers.
Cell line cultures

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and were used with no more than 20 passages.

i) Human ACC cell line NCI-H295R (ATCC, CRL-2128) was cultured as a monolayer in DMEM/Ham’s F12 medium supplemented with 6.25 μg/ml transferrin, 6.25 μg/ml insulin, 6.25 ng/ml selenium, 5.35 μg/ml linoleic acid, 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37 °C and harvested weekly. The most secreted steroids are cortisol and DHEAS (Rainey et al. 2004) by stimulation with dibutyryl cAMP (db-cAMP) or forskolin or with ACTH to a lesser extent. For this reason, steriodogenesis was stimulated in the presence of db-cAMP. In addition, aldosterone concentrations were determined by stimulation with 10–7 M angiotensin II in the absence or presence of HA at two different concentrations (10–5 or 10–9 M). Cortisol and DHEAS concentrations were measured by RIA using commercial kits (Coat-a-Count; Siemens Healthcare Diagnostic, Los Angeles, CA, USA). Aldosterone concentrations were quantified as described previously (Mele et al. 2012).

ii) Y1 cells (ATCC, CCL-79) are an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line (Yasumura 1968) and mainly produce progesterone. The cells were grown as a monolayer in Ham’s F10 medium containing heat-inactivated fetal bovine serum and horse serum (2.5 and 12.5% respectively), 200 U/ml penicillin G, and 270 μg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO2 in air at 37 °C and harvested weekly. The most secreted steroids are cortisol and DHEAS (Rainey et al. 2004) by stimulation with dibutyryl cAMP (db-cAMP) or forskolin or with ACTH to a lesser extent. For this reason, steriodogenesis was stimulated in the presence of db-cAMP. In addition, aldosterone concentrations were determined by stimulation with 10–7 M angiotensin II in the absence or presence of HA at two different concentrations (10–5 or 10–9 M). Cortisol and DHEAS concentrations were measured by RIA using commercial kits (Coat-a-Count; Siemens Healthcare Diagnostic, Los Angeles, CA, USA). Aldosterone concentrations were quantified as described previously (Mele et al. 2012).

Determination of intracellular cAMP production

The cells were seeded in 24-well microplates (5 × 10^5 cells/well) in the complete medium. After 24 h, the medium was replaced with the assay medium (DMEM/Ham’s F12 and 0.1% BSA). After 20-min incubation with the corresponding stimulus (10–5 M HA, 10–5 M FMPH, or 5 × 10–3 M forskolin), the cells were extracted with 0.5 ml of cold ethanol. After centrifugation for 15 min at 9000 g, supernatants were evaporated and pellets were resuspended using 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and assayed by RIA as described previously (Del Punta et al. 1996). The inter-assay and intra-assay CV values were lower than 10%.

Determination of [3H]-inositol phosphate production

The cells were incubated in a six-well microplate (1 × 10^6 cells/well) with 2 μCi of myo-[3H]-inositol for 48 h before the experiment. At the end of the labeling period, the cells were washed with the assay medium (DMEM/Ham’s F12 and 0.1% BSA) and preincubated for 15 min with 20 mM LiCl. At the end of this period, 10–5 M HA, 10–5 M FMPH, or 10–3 M NaF (positive control) was added. After 30-min incubation, total inositol phosphate (InsPn) was measured as described previously (Ascoli et al. 1989) using Dowex columns.

Results are expressed as the ratio obtained when [3H]-InsPn activity was normalized to total [3H]-inositol recovered from the initial wash of the Dowex columns corresponding to the intracellular [3H]-inositol pool (Mondillo et al. 2005).

Ligand binding assays for HRH1 subtype

The cells were seeded in 24-well microplates (5 × 10^5 cells/well) and cultured for 48 h in the complete medium. The cells were rinsed twice with PBS and incubated for 40 min at 4 °C in 200 ml of 50 mM Tris/HCl (pH 7.5) containing increasing concentrations of [3H]-pyrilamine (1–1000 nM). Nonspecific binding was defined with 100 mM cold pyrilamine. After incubation, the cells were washed with ice-cold 50 mM Tris/HCl at 4 °C and scraped from the wells; radioactivity was determined by liquid scintillation counting.

[3H]-Thymidine incorporation assay

DNA synthesis was evaluated according to the amount of [3H]-thymidine incorporated into the H295R cells. The cells were seeded in 96-well microplates (3 × 10^4 cells/well) in the complete medium. After 18 h, the medium was replaced with DMEM/F12 medium with reduced serum (1%) and incubated with different concentrations of HA and the indicated compounds for 24 h, with a pulse of 0.25 μCi/ml [3H]-thymidine during the last 12 h. At the end of the pulse period, the cells were frozen at –20°C and harvested in glass fiber discs by filtration. The samples
were washed with 95% ethanol, dried, and counted by liquid scintillation counting.

**MTT assay**

The MTT assay is based on the transformation and colorimetric quantification of MTT. In this assay, a linear relationship between cell number and signal produced is established, thus allowing for the quantification of cell proliferation. In brief, the cells were plated in 96-well microplates (3×10^4 cells/well), and they were treated with the indicated compounds 24 h later. After 24 h, MTT was added (final concentration 0.5 mg/ml), and the cells were incubated at 37 °C for 2 h. To stop the coloring reaction and dissolve the formed formazan crystals, a solubilization solution (isopropanol with HCl) was added, and the mixture was incubated overnight at room temperature. Color intensity was measured at 570 nm using a multiplate ELISA reader.

**Cell-cycle analysis**

H295R cells were seeded in six-well microplates (1.5×10^6 cells/well) in the complete medium. After 18 h, the medium was replaced with DMEM/F12 medium with reduced serum (1%) and the cells were incubated with HA or FMPH, both at a concentration of 10^{-5} M, for 24 h. After the incubation period, the cells were harvested by trypsination, centrifuged, washed twice in PBS, and fixed in PBS:ice-cold ethanol (1:3). After centrifugation at 37 °C, the cells were finally resuspended in 3.8 mM sodium citrate buffer, containing 40 μg/ml PI and 100 μg/ml DNase–free RNase A. After 30 min incubation, the samples were measured with a FACSAria flow cytometer. The percentage of cells in the G1, S, and G2/M phases of the cell cycle were determined with WinMDI 2.8 (Joe Trotter, WinMDI, Scripps Institute, La Jolla, CA, USA) and Cylchred analytical software (Terry Hoy, Cylchred, Cardiff University, UK).

**TUNEL assay**

H295R cells were cultured in six-well microplates (1.5×10^6 cells/well) in the complete medium. After 18 h, the medium was replaced with DMEM/F12 medium with reduced serum (1%), and the cells were incubated with HA or FMPH, both at a concentration of 10^{-5} M, for 24 or 72 h. After incubation, nuclear DNA fragmentation was detected by the TUNEL method using the cell death detection kit (Roche Applied Science) according to the manufacturer’s instructions. Apoptosis was analyzed by flow cytometry, and data were processed with WinMDI 2.8 Software.

**Western blot analysis**

Total cell protein was obtained by placing cells in a lysis buffer (10 mM Tris–HCl, 1% Triton X-100, and 0.5 mM EGTA, pH 7.4) containing a protease inhibitor cocktail (5 μg/ml leupeptin, 25 mM NaF, 25 mM sodium orthovanadate, 400 μM phenylmethylsulphonyl fluoride, 5 μg/ml pepstatin, and 5 μg/ml aprotinin), followed by 30 passages through a 1 ml syringe. Protein concentrations were measured using the Bradford assay for total protein. Equal amounts of protein per sample (50 μg) were loaded onto a 10% (w/v) SDS–polyacrylamide gel (Mini Protean III System; Bio-Rad). Electrophoresis, transfer of proteins onto PVDF membranes, and immunodetection of STAR, HDC, caspase-3, and β-tubulin were performed under optimized conditions.

**Immunocytochemistry of HA content**

Y1 and H295R cells were seeded on 12 mm-diameter round glass coverslips, precoated with polylysine (3×10^4 cells/coverslip), and washed and fixed with 4% formaldehyde for 15 min at room temperature after 3 days. The cells were permeabilized for 10 min with 0.25% Triton X-100 and 0.3 M glycine in PBS (PBST), and unspecific binding was blocked with 1% BSA in PBST for 30 min. The coverslips were incubated for 24 h with primary antibody against HA (1:100) or normal rabbit serum in PBS (negative control) overnight at 4 °C, followed by incubation with a secondary conjugated anti-rabbit antibody coupled to peroxidase (1:4000) for 1.5 h at room temperature. Immunoreactivity was detected with 2.7 mM 3,3-diaminobenzidine tetrahydrochloride in PBS with 0.03% hydrogen peroxide (w/v). For the quantification of HA immunocytochemical staining, representative cells were chosen and visualized by 40×10 magnification through Zeiss-Axioskop (Zeiss Oberkochen, Germany) with an Olympus DP70 digital camera. For each cell type, 500 cells in three independent experiments were subjected to histogram analysis using Photoshop CS 8.0.1. Mean gray values from negative controls were subtracted from mean gray values determined from cells stained for HA to exclude background staining.

**Statistical analysis**

All the experiments were repeated at least three times. If heterogeneity of variance was detected by Bartlett’s test, it was reduced by logarithmic transformation of the data before analysis. These data were then subjected...
to Student’s t-test or one-way ANOVA followed by Bonferroni’s test for multiple range comparisons. P values <0.05 were accepted as significant.

Results

Effect of HA on steroid production and STAR expression in Y1 and H295R adrenocortical cells

Y1 and H295R cells were incubated with increasing concentrations of HA (10^{-11}-10^{-5} M) for 5 or 24 h in the absence or in the presence of 1 mIU/ml ACTH or 0.5 mM db-cAMP for each cell line respectively. Figure 1A and B show that HA treatment did not modify steroid synthesis in any cell type, unstimulated or stimulated, at any HA concentration or incubation time period. The figure only shows results obtained for 24-h treatment and cortisol quantification in H295R cells. As has been mentioned previously, DHEAS and aldosterone concentrations were also measured, but no differences were observed. Aldosterone production was increased after stimulation with angiotensin II, but different HA concentrations did not modify steroidogenesis.

To exclude a possible simultaneous activation of different receptors with antagonistic effects, steroid production was assessed for 24 h in the presence of different agonists specific for each receptor subtype in the absence or presence of stimulus (1 mIU/ml ACTH for Y1 cells or 0.5 mM db-cAMP for H295R cells). Compounds used were as follows: FMPH as the HRH1 agonist, AMTH as the HRH2 agonist, IMET as the HRH3 agonist, and VUF as the HRH4 agonist, all at a concentration of 10^{-5} M, which we have used previously (Mondillo et al. 2009, Medina et al. 2011, Pagotto et al. 2012). As can be seen in Fig. 1C and D, there were no differences in steroid production under any treatment condition with respect to the control values.

STAR is a protein that mediates the rate-limiting step in steroid hormone biosynthesis (Stocco & Clark 1996). As we had already demonstrated that HA decreases the levels of STAR protein in LCs (Mondillo et al. 2009), we studied the effect of HA on STAR expression in adrenocortical cells.

Y1 and H295R cells were incubated for different time periods in the absence or presence of 10^{-5} M HA and in the presence of 1 mIU/ml ACTH or 0.5 mM db-cAMP for each cell line respectively. HA concentration (10^{-5} M) was the same as that we had used previously for the treatment of MA-10 LCs, showing a marked reduction in db-cAMP-stimulated STAR protein expression (Mondillo et al. 2009). In contrast to LCs, but in concordance with results obtained for adrenal steroidogenesis, HA did not modify STAR protein expression at any time period with respect to the controls either in Y1 cells (Fig. 2A) or in H295R cells (Fig. 2B). The progressive increase in STAR expression in both cell lines treated with their respective stimuli is in agreement with results described in previous reports of our group (Piotrkowski et al. 2009) and others (Manna et al. 2009).

Effect of HA on proliferation in Y1 and H295R adrenocortical cells

To evaluate the effect of HA on cell proliferation, we carried out [3H]-thymidine incorporation assay. Different results were obtained for the two cell lines: HA did not modify Y1 cell proliferation (Fig. 3A), but a concentration-dependent inhibition of H295R cell proliferation was observed (Fig. 3B) with a maximal effect at 10^{-5} M (32.6% inhibition with respect to the control). Complete medium (5% FCS) was used as a positive control.
To assess which HA receptor subtype/s could be involved, cell proliferation was studied in the presence of specific HA agonists, all at a concentration of $10^{-5}$ M. As has been described previously, in the presence of HA, treatment with agonists did not modify $[^3H]$-thymidine incorporation in Y1 cells (Fig. 3C). In contrast, in H295R cells, the HRH1 agonist FMPH inhibited the proliferation to an extent similar to that observed in the presence of $10^{-5}$ M HA (35.7%; Fig. 3D).

To confirm the HA-mediated inhibitory effect through HRH1 on cell proliferation, H295R cells were preincubated for 30 min with $10^{-8}$ M pyrilamine (specific antagonist for HRH1). The cells were then incubated with HA or FMPH ($10^{-5}$ M), and $[^3H]$-thymidine incorporation was determined. Figure 4A shows that pyrilamine treatment reversed the inhibitory effects of HA and FMPH on cell proliferation, but had no effect when cells were incubated alone. These results were corroborated using the MTT assay as an alternative method to measure cell proliferation (Fig. 4B).

To exclude a nonspecific toxic effect of the tested compounds on H295R cells, cell viability was evaluated using PI staining for the detection of nonviable cells by flow cytometry. As no differences in cell viability were found between treatments at the evaluated time period (data not shown), a toxic effect of HA was excluded.

Figure 2
Effect of HA on STAR protein expression. Y1 and H295R adrenocortical cells were incubated in the presence or absence of $10^{-5}$ M HA under stimulated steroidogenesis, for different time periods, as described in the ‘Materials and methods’ section. After incubation, proteins were extracted, and the expression of STAR protein was analyzed by western blot analysis. Data were normalized to those the internal control $\beta$-tubulin. (A and B) Representative western blots of STAR protein in Y1 and H295R cells respectively. (C and D) Quantitation of STAR protein levels by scanning densitometry in Y1 and H295R cells respectively. Each bar shows the means $\pm$ S.E.M. of three independent experiments carried out with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P<0.05$.

Figure 3
Effects of HA and its specific agonists on adrenocortical cell proliferation. The murine cell line Y1 and the human cell line H295R were incubated with increasing concentrations of HA (A and B respectively) or agonists specific for each HA subtype receptor, FMPH (HRH1), AMTH (HRH2), IMET (HRH3), and VUF (HRH4) (C and D respectively), at a concentration of $10^{-5}$ M, for 24 h. The cells were then incubated with HA or FMPH ($10^{-5}$ M), and $[^3H]$-thymidine incorporation was determined. Figure 4A shows that pyrilamine treatment reversed the inhibitory effects of HA and FMPH on cell proliferation, but had no effect when cells were incubated alone. These results were corroborated using the MTT assay as an alternative method to measure cell proliferation (Fig. 4B). To exclude a nonspecific toxic effect of the tested compounds on H295R cells, cell viability was evaluated using PI staining for the detection of nonviable cells by flow cytometry. As no differences in cell viability were found between treatments at the evaluated time period (data not shown), a toxic effect of HA was excluded.
Characterization of HRH1 in H295R adrenocortical cells

As HA inhibited H295R cell proliferation through HRH1, we aimed to further characterize this receptor subtype in the cell line. A saturation binding assay was carried out using [3H]-pyrilamine as a specific ligand (Fig. 5B). The nonlinear regression best fitted a one-site model, indicating the presence of a single class of sites for HRH1 in H295R cells with a $K_d$ value of 124.4 ± 15.8 nM (95% CI, 93.52–155.2) and a $B_{\text{max}}$ value of 4.0 ± 0.2 fmol/mg protein (95% CI, 3.7–4.4). The same assay was carried out in Y1 cells for comparison (Fig. 5A). The binding to intact Y1 cells also best fitted a one-site model and a single class of sites with a $K_d$ value of 21.8 ± 8.3 nM.

Figure 4
Effects of the HRH1 antagonist pyrilamine on HA-mediated H295R cell proliferation. H295R cells were preincubated with $10^{-8}$ M pyrilamine and HA, FMPH, or medium was added to the culture after 30 min and incubated for 24 h. (A) Cell proliferation was measured by [3H]-thymidine incorporation into DNA as described in the ‘Materials and methods’ section. (B) Cell proliferation was measured by MTT assay. The cells were incubated with 0.5 mg/ml MTT and OD was recorded at 570 nm. Cell number was calculated using a linear relation between OD values and cell number. Data are expressed as proliferation percentages with respect to the control (cells incubated without HA; dashed line). Bars represent the means ± S.E.M. of at least three independent experiments. *$P<0.05$ vs control and **$P<0.01$ vs control.

Figure 5
Binding assay for HRH1 in adrenocortical cell lines. Saturation binding assays were carried out on intact Y1 cells (A) and H295R cells (B) using [3H]-pyrilamine as a ligand specific for HRH1. Saturation analysis revealed a single and saturable binding site in both cell lines. In the insets, Scatchard plots of [3H]-pyrilamine-specific binding are shown. A typical result of an experiment replicated three times, with data representing mean of duplicate determinations for each cell line, is shown. Bars represent s.e.m.
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(95% CI, 4.8–38.8) and a $B_{\text{max}}$ value of $1.2 \pm 0.2$ fmol/mg protein (95% CI, 0.8–1.5).

**Signaling pathway of the HA anti-proliferative effect in the H295R cell line**

To evaluate the signaling pathway activated by HRH1 in H295R cells, cAMP and InsP$_n$ levels were measured in cells incubated with HA and FMPH, the specific HRH1 agonist. Forskolin and NaF were used as positive controls for each second messenger respectively.

cAMP levels were not modified by either HA or FMPH, while both these compounds produced a twofold increase in the total InsP$_n$ content of H295R cells above the basal level (Fig. 6A and B respectively). Furthermore, blockage of PLC using the specific inhibitor U-73122 in the presence of FMPH prevented the decrease in $[^{3}\text{H}]$-thymidine incorporation observed with the HRH1 agonist alone, whereas U-73343, a nonfunctional inhibitor analog of U-73122,

**Figure 6**

Signaling pathway of HA anti-proliferative effect in H295R cells. (A) Intracellular cAMP production. The cells were incubated for 20 min with $10^{-5} \text{ M HA}, 10^{-5} \text{ M FMPH}$, or $5 \times 10^{-4} \text{ M forskolin (Forsk)}$, used as a positive control. cAMP levels were measured by RIA. (B) Total $[^{3}\text{H}]$-inositol phosphate accumulation. The cells preincubated with $[^{3}\text{H}]$-myo-inositol were treated with $10^{-5} \text{ M HA}, 10^{-5} \text{ M FMPH}$, or $10^{-3} \text{ M NaF}$, used as a positive control. Total $[^{3}\text{H}]$-inositol phosphate content was quantified by recovered radioactivity, as described in the 'Materials and methods' section. Bars represent the means $\pm$ S.E.M. of at least three independent experiments. Different letters above the bars indicate that the groups differ significantly (at least $P<0.01$). (C) Involvement of phospholipase C in H295R cell proliferation. H295R cells were incubated with the specific PLC inhibitor U-73122 or its nonfunctional analog U-73343 in the presence of FMPH for 24 h and proliferation was determined by $[^{3}\text{H}]$-thymidine incorporation into DNA during the last 12 h of the incubation period, as described in the 'Materials and methods' section. Data are expressed as proliferation percentage with respect to the control (cells incubated without HA; dashed line). Bars represent the means $\pm$ S.E.M. of at least three independent experiments. $^*P<0.05$ vs control.

**Figure 7**

Effect of HA and FMPH on H295R cell apoptosis. (A) Evaluation of apoptosis by TUNEL assay. H295R cells were incubated with HA or FMPH, both at a concentration of $10^{-5} \text{ M}$, for 24 h, processed by TUNEL assay, and analyzed by flow cytometry as described in the ‘Materials and methods’ section. Doxorubicin was used as a positive control for apoptosis. Bars represent the means $\pm$ S.E.M. of three independent experiments. $^*P<0.01$ vs control and $^{**}P<0.001$ vs control (B) Evaluation of caspase-3 activation by western blot analysis. H295R cells were incubated with HA or FMPH (both at a concentration of $10^{-5} \text{ M}$) for 0, 6, 18, 24, 48, and 72 h. Proteins were extracted and subjected to SDS–PAGE. Caspase-3 was detected using a specific antibody in both forms, inactive precursor (molecular weight 32) and active subunits (molecular weights 17 and 11). Active subunits of caspase-3 were not detected even after 72 h of treatment with HA or FMPH in H295R cells. EDS-treated MA-10 cells were used as control for anti caspase-3 antibody.
was not able to block the FMPH-induced anti-proliferative effect (Fig. 6C).

**Effect of HA on apoptosis and cell-cycle control in H295R adrenocortical cells**

To determine whether the growth-inhibitory effect of HA on H295R cells affected apoptosis, the cells were cultured with HA or FMPH (10^{-5} M) for 24 and 72 h, and apoptosis was evaluated by TUNEL assay using flow cytometry. As shown in Fig. 7A, apoptosis levels were not different between the treated and control cells, doxorubicin (an apoptosis inducer) significantly increased the proportion of apoptotic cells in a concentration-dependent manner. For simplicity, Fig. 7 shows only results obtained for 24-h treatment. Similar results were obtained for the 72-h incubation period.

To confirm the above results, the presence of activated caspase-3 (an apoptosis marker) was evaluated by western blot analysis using protein extracts from H295R cells incubated with HA or FMPH (10^{-5} M) at different time periods (0, 6, 18, 24, 48, and 72 h). Bands corresponding to the cleaved forms of caspase-3 (corresponding to molecular weights of 17 and 11) were not detected by immunoblotting at any time period analyzed (Fig. 7B).

The effect of HA on cell-cycle progression was examined later. H295R cells were treated with 10^{-5} M HA or 10^{-5} M FMPH for 24 h, and cell-cycle distribution was analyzed using flow cytometry and PI staining (Fig. 8A). Figure 8B shows a significant increase in the

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**Figure 8**

Effect of HA and FMPH on H295R cell-cycle progression. H295R cells were incubated with HA or FMPH, both at a concentration 10^{-5} M, for 24 h. After incubation, the cells were fixed, permeabilized, and stained with propidium iodide as described in the 'Materials and methods' section. DNA content was analyzed by flow cytometry. (A) Histogram of DNA content for each treatment, from representative experiments. (B) H295R cell percentage distribution in G1/G0, G2/M, and S cell-cycle phases from all experiments. Bars represent the means±S.E.M. of three independent experiments. *P<0.05 vs control.
percentage of cells in the G2/M phase when they had been treated with both HA and FMPH (in percentages of cells: Control, 4.6\% ; HA, 8.75\% ; and FMPH, 10.9\% ), with a concomitant decrease in the proportion of cells in the S phase (in percentages of cells: Control, 43.0\% ; HA, 28.6\% ; and FMPH, 31.2\% ).

**Expression of HDC enzyme and endogenous HA content in the H295R cell line**

In an attempt to find a possible explanation for the differential effects of HA on the proliferation of H295R vs Y1 cells, and considering the well-documented correlation between HDC expression and cell proliferation in several experimental models (Falus et al. 2011), we aimed to compare the expression levels of HDC enzyme in the two cell lines by western blot analysis. As shown in Fig. 9A, the active form of HDC enzyme (molecular weight 53–55) was expressed at significantly higher levels in Y1 cells than in H295R cells. Stomach was used as a positive control. Coincidentally, the endogenous content of HA revealed by immunocytochemistry and quantified as described in the ‘Materials and methods’ section was higher in Y1 cells (at least $P<0.05$). (B, upper panel) Immunocytochemical staining of endogenous HA content in Y1 and H295R cell lines. As a negative control, the primary antibody was replaced with normal rabbit serum in PBS. Scale bar=50 µm. (B, lower panel) Quantitation of HA content by scanning densitometry in arbitrary units (AU). Each bar shows the means\(\pm\)S.E.M. of three independent experiments; for each cell line, 500 cells were analyzed. Different letters above the bars indicate that the groups differ significantly (at least $P<0.05$).

**Figure 9**

HDC protein expression and endogenous HA content in adrenocortical cell lines. Y1 and H295R cells were lysed and subjected to western blot analysis for the detection of HDC protein, as described in the ‘Materials and methods’ section. Data were normalized to those of the internal control $\beta$-tubulin. (A, upper panel) Representative western blot of HDC protein. Rat stomach was used as a positive control. (A, lower panel) Quantitation of protein levels by scanning densitometry. Each bar shows the means\(\pm\)S.E.M. of three independent experiments carried out with triplicate samples. Different letters above the bars indicate that the groups differ significantly (at least $P<0.05$).

As an approach to demonstrating more directly the role of HDC and HA content in Y1 and H295R cell proliferation, we evaluated the effect of the catechin EGCG, known to potently inhibit HDC activity, as has been recently shown by us (Pagotto et al. 2012) and others (Nitta et al. 2007, Ruiz-Perez et al. 2012), on the proliferation of Y1 cells. As can be seen in Fig. 10, EGCG inhibited Y1 cell proliferation in a concentration-dependent manner. EGCG concentrations higher than $4\times10^{-5}$ M were found to be toxic.

**Discussion**

The existence of a functional histaminergic system in the testis of different species has been demonstrated previously by us (Mondillo et al. 2005, 2007, 2009, Pagotto et al. 2012) and others (Mayerhofer et al. 1989, Pap et al. 2002, Albrecht et al. 2005, Khan & Rai 2007). Particularly, we have reported that low concentrations of HA ($10^{-9}$ M) stimulate LC steroidogenesis and higher concentrations...
(10^{-5} \text{ M})$ inhibit it (Mondillo et al. 2005). To assess whether the effects of HA on steroid synthesis could be extrapolated to other steriodogenic systems, we studied the direct action of the amine and its agonists on steroidogenesis in Y1 and H295R adrenocortical cells, two well-documented cell lines used for the study of adrenal cortex function. Considering that adrenal steroids and steroidogenesis regulation vary among species and as H295R cells are of human origin and Y1 is a murine cell line, we evaluated the production of major steroids in each cell line and the biosynthetic rate-limiting-step enzyme STAR. In this respect, no significant effect was found on steroid production or enzyme-associated expression under any of the evaluated conditions.

These results are in agreement with those of previous research using bovine co-cultures of adrenal medulla and cortex cells, in which an indirect effect of HA through HRH1 present in adrenal medulla has been observed, so inducing the release of neuropeptides that would act on adrenocortical cells, regulating the secretion of cortisol (Yoshida et al. 1997, Ehrhart-Bornstein et al. 1998, 2000). In the present study, we used adrenocortical cell lines excluding contamination with chromaffin cells. Then, according to our observations on Y1 and H295R cells, HA would not be able to directly regulate steroid synthesis in adrenocortical cells, showing that the modulatory effect observed in LCs cannot be extrapolated to all steriodogenic systems.

Regarding the ability of HA to regulate adrenocortical cell proliferation, results were different between the tumor cell lines evaluated. The Y1 murine cell line did not respond to HA treatment, whereas the H295R cell line on treatment with $10^{-5} \text{ M}$ HA exhibited growth that was reduced to about 60% of that observed in the control. This effect was reproduced by FMPH, an agonist specific for HRH1.

In bovine adrenal glands, the expression of HRH1 in medulla and cortex at different levels and affinities, with both being higher in the medulla, has been reported (Chang et al. 1979, Yamashita et al. 1991). Herein, the presence of a functional HRH1 in the human ACC cell line H295R is reported.

The differential effect of HA on H295R and Y1 cell proliferation could be explained, at least in part, if Y1 cells exhibited higher expression levels of HDC enzyme and endogenous HA content than H295R cells. To test this hypothesis, HDC enzyme from Y1 cells was inhibited and proliferation was measured. In fact, inhibition of proliferation was observed, indicating that the higher HA content in Y1 cells prevents the inhibition observed in H295R cells in the presence of exogenous HA. The endogenous HA content sustained over time could have triggered the internalization of its receptors, as reported in other systems, canceling responsiveness to HA (Miyoshi et al. 2006, Hishinuma et al. 2010). The lower number of HRH1 in Y1 cells than in H295R cells, calculated using the Scatchard analysis (1.2 vs 4.0 fmol/mg protein respectively), supports this hypothesis.

There is growing evidence that HA can negatively modulate cell proliferation in diverse systems through the activation of different receptor subtypes, e.g. HRH1 (Valencia et al. 2001), HRH2 (Cricco et al. 2006), HRH3 (Francis et al. 2009), and HRH4 (Meng et al. 2011).

In humans specifically, the influence of HA on adrenocortical cells had already been suggested by Szabó et al. (2009), who compared HA-related gene expression in normal and tumoral adrenal cortex tissues. They found not only differential expression patterns for HA receptor subtypes in ACC but also a reduction in HDC expression level and HA content compared with normal tissues. These observations are in agreement with the results of the present study, in which the addition of HA was able to inhibit proliferation in H295R cells.

As has been mentioned previously, HA-mediated growth inhibition in H295R cells was induced by the activation of HRH1, with an increase in InsP3 levels,
indicating that in adrenocarcinoma cells the activation of HRH1 would be associated with the classic signaling pathway involving a PLC. The reversion of the HA anti-proliferative effect in the presence of the specific PLC inhibitor U-73122 confirmed these results.

A similar HA anti-proliferative signaling mechanism has been described for the prostate cancer cell line DU-145 (Valencia et al. 2001) as well as CHO cells stably transfected with HRH1, where HA activates a PLC, leading to an inhibition of proliferation through a mechanism mediated by GTPase, Rac, and c-Jun-kinase (Notcovich et al. 2010). It is known that angiotensin II stimulates aldosterone production in H295R cells through AT1 receptor coupled to PLC, increasing the production of InsPn (Rainey et al. 2004). Although HA inhibited H295R cell proliferation by increasing InsPn levels without activating aldosterone production, it is possible that HA stimulates NOS enzyme activity (via Ca2+), blocking steroidogenesis as described previously for MA-10 LCs by us (Mondillo et al. 2009) and has been observed in other steroidogenic systems (Ducsay & Myers 2011). Regarding this, it has been demonstrated that NOS can inhibit L-type calcium channels (Wang et al. 2008), which are necessary for all-mediated steroidogenesis. Supposing that HA induced NOS in H295R cells, the entry of calcium through the L-channels would be blocked, thus preventing aldosterone synthesis, without affecting the proliferation pathway. Nevertheless, activation of other kinase signaling pathways by other HA receptors, with an antagonizing effect, cannot be excluded.

The present study demonstrated that treatment with HA or FMPH, the HRH1 agonist, is capable of inhibiting cell proliferation in human adrenocortical tumor cells in vitro without inducing apoptosis, as confirmed by the TUNEL and caspase-3 immunoblot assays. In addition, treatment with HA or FMPH induced a cell-cycle arrest in the H295R cell line in the G2/M phase. Transition between cell-cycle phases is a process that relies on the formation of cyclin–cyclin-dependent kinase complexes as well as their interaction with specific inhibitors.

Several proteins have been reported to be associated with the control of entry into the G2/M phase (Smits & Medema 2001). In this regard, in H295R cells, a G2/M phase arrest induced by combinatory treatment with mitotane and ionizing radiations has been described. These agents act by attenuating the DNA repair mechanisms and maintaining high levels of cyclin B1–Cdc2 complexes (Cerquetti et al. 2010). It is likely that at least some of these events are involved in the G2/M phase arrest induced by HA. Further studies need to be conducted to confirm this hypothesis.

Currently, nonsurgical treatments for human ACC are scarce and based on ionizing radiations in association with high doses of adrenolytic drugs, bringing about toxic side effects that limit their usefulness (Maluf et al. 2011). Our results indicate that HA would exert a cytostatic effect on H295R cells, arresting cell growth in a DNA damaging-sensitive phase (G2/M), without inducing death. Future studies need to be carried out to evaluate whether these features could make HA a good candidate for new ACC therapies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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