HISTAMINE INHIBITS ADRENOCORTICAL CELLS PROLIFERATION BUT DOES NOT AFFECT STEROIDOGENESIS

PAGOTTO, ROMINA MARIA¹,³; PEREYRA, ELBA NORA¹; MONZÓN, CASANDRA¹; MONDILLO, CAROLINA¹; PIGNATARO, OMAR PEDRO¹,²

1 Laboratory of Molecular Endocrinology and Signal Transduction, Institute of Biology and Experimental Medicine, National Research Council (IByME-CONICET), CP 1428, Buenos Aires, Argentina; 2 Department of Biological Chemistry, School of Sciences, University of Buenos Aires (UBA), CP 1428, Buenos Aires, Argentina; 3 Present address: Cell Biology Unit, Institut Pasteur de Montevideo, CP11400, Montevideo, Uruguay

Correspondence and reprint requests should be addressed to:
Omar P. Pignataro, MS, PhD
Laboratory of Molecular Endocrinology and Signal Transduction
Institute of Biology and Experimental Medicine (IByME-CONICET)
Vuelta de Obligado 2490
CP 1428
Buenos Aires, Argentina.
FAX: 54 011 4786 2564
e-mail: oppignataro@gmail.com

Short title: Histamine inhibits adrenal cell proliferation

Key words: Histamine, adrenocortical cells, proliferation, steroidogenesis

Word count: 5069
2. 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 (LC) have been recently described. In order to determine if the effects reported in LC could be extrapolated to all steroidogenic systems, we studied the effect of this amine on proliferation and steroidogenesis of the adrenal cortex, using two adrenocortical cell lines as experimental models, the murine Y1 and the 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 the HRH1 receptor subtype and an increase in the production of inositol phosphates as second messengers, causing a cell cycle arrest in the G2/M phase. These results indicate a new role of HA on human adrenocortical cells proliferation that could contribute to a better understanding of tumor pathology as well as to the development of new therapeutic agents.
3. Introduction

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

As regards steroidogenesis, HA was shown to stimulate this process 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 and in purified rat Leydig cells (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 have also shown that NOS activation is the main intracellular mechanism by which HA exerts its anti-steroidogenic effects (Mondillo, et al. 2009).

Over the last years, proliferative actions of HA have become more relevant 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 anti-mitogenic (Cricco, et al. 2006; Meng, et al. 2011; Petit-Bertron, et al. 2009) and mitogenic agent (Francis, et al. 2009; Medina, et al. 2011; Molina-Hernandez and Velasco 2008; Stoyanov, et al. 2012) depending on the cell type and the HA receptor pattern expressed. Particularly, our recent studies showed, for the first time, the proliferative effect of HA in MA-10 Leydig cells, mediated via HRH2 activation, 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 rat and dog by acting on CNS via the HRH1 by an 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 the HRH1, stimulating the secretion of catecholamines and neuropeptides which, in turn, act in a paracrine way on cortical cells regulating adrenal cortisol secretion (Bunn and Boyd 1992). Concerning the presence of HA in the adrenal gland, it has been identified in guinea pig and rat adrenal glands, most being present in the cortex (Endo and Ogura 1974). Possible sources of cortical HA would be those from subpopulations of adult chromaffin cells.
present in the medulla (Tuominen, et al. 1993), the endings of the splanchnic nerve and the contribution of mast
cells that are arranged surrounding adrenal arterioles, near the capsule (Borges 1994; Hinson, et al. 1989). 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 they come
from studies on different species and utilize 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 refer to a
direct effect of HA on cortisol secretion (Aikawa, et al. 1986; Matsumoto, et al. 1981) 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 have been reported that include the study of a possible direct action of
HA on pure adrenocortical cell lines, which would define the situation unequivocally.
About human adrenocortical proliferation and HA, Szabó et al (Szabo, et al. 2009) have recently published that
HDC expression and HA content were highest in normal tissues, lower in benign tumors, and significantly lower
in Adrenocortical Carcinoma (ACC).
Considering the information above and our previous findings about the ability of HA to regulate testicular
steroidogenesis, the aim of this work was to study the direct effect of this amine on adrenal steroidogenesis and
proliferation. To reach 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
4. Materials and Methods

4.1. 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 VUF8430 (VUF), TME-cAMP, BSA, MTT, transferrin, selenium, glutamine and NaHCO₃, phospholipase C inhibitor (U-73122), PLC inactive analog inhibitor (U-73343), doxorubicin, epigallocatechin gallate (EGCG), mouse monoclonal anti-β tubulin and caspase-3 antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]-Pyrilamine, Na[125I]-I, [3H]-Thymidine and Myo-[3H]-inositol were purchased from NEN (Boston, MA).

Cell culture supplies were from Gibco-BRL (Gaithersburg, MD). Dowex AG-I-X8 resin was from Bio-Rad (Hercules, CA). TME-cAMP was radiolabeled with Na¹²⁵I in our laboratory by the method of chloramine-T (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 from Alpha Diagnostic (San Antonio, USA). Rabbit anti-HDC antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Secondary conjugated anti-rabbit antibody coupled with peroxidase was from Vector Labs (Burlingame, USA). DMEM/F12 medium (GIBCO) and Hyclone supplemented calf serum (Thermo Scientific) were 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 commonly used suppliers.

4.2. Cell line cultures

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

1- Human adrenocortical cancer cell line NCI-H295R (ATCC, CRL-2128) was cultured as 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 mostly
secreted steroids are cortisol and dehydroepiandrosterone sulfate (DHEAS) (Rainey et al, 2004) by stimulation with db-cAMP or forskolin or with ACTH at a lesser extent. For this reason, stimulation of steroidogenesis was done in the presence of db-cAMP. In addition, aldosterone was determined by stimulation with $10^{-7}$ M Angiotensin II, in the absence or presence of two different concentrations of HA ($10^{-5}$ or $10^{-9}$ M). Cortisol and DHEAS were measured by RIA with commercial kits (Coat-a-Count, Siemens Healthcare Diagnostic, LA, USA). Aldosterona was quantified as described (Mele, et al. 2012).

2- Y1 cells (ATCC, CCL-79) are an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line (Yasumura 1968) and produce mainly progesterone. Cells were grown as monolayer in Ham’s F10 medium containing heat-inactivated fetal bovine and horse serum (2.5% and 12.5% respectively), 200 U/ml penicillin G, and 270 µg/ml streptomycin sulphate, in a humidified atmosphere of 5% CO$_2$ in air at 37°C. Progesterone was measured by RIA as previously described (Piotrkowski, et al. 2009). The intra and inter-assay variations were 8.0% and 14.2% respectively.

### 4.3. Determination of intracellular cAMP production

Cells were seeded in 24 well microplates (5 x $10^5$ cell/well) in complete medium. After 24 h, media 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 x $10^{-3}$ M forskolin) cells extracted with 0.5 ml of cold ethanol. After centrifugation for 15 min at 9000 x 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 (Del Punta, et al. 1996). The inter and intra-assay variations of coefficients were lower than 10%.

### 4.4. Determination of [3H]-Inositol Phosphates production

Cells were incubated in a 6 well microplate (1 x $10^6$ cell/well) with 2 µCi of myo-[3H]-inositol for 48 h before the experiment. At the end of the labeling period, cells were washed with 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 (as positive control) was added. After 30 min incubation, total inositol phosphates (InsPn) were measured as previously described (Ascoli, et al. 1989) by using Dowex columns.

Results were 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).

4.5. Ligand binding assays for HRH1 subtype histamine receptor

Cells were seeded in 24 well microplates (5 x 10^5 cell/well) and cultured for 48 h in 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 to 1000 nM). Nonspecific binding was defined with 100 mM cold pyrilamine. After incubation, cells were washed with ice-cold Tris/HCl 50 mM at 4°C and scraped to remove them from the wells; radioactivity was determined by liquid-scintillation counting.

4.6. [3H]-Thymidine incorporation assay

DNA synthesis was evaluated according to the amount of [3H]-Thymidine incorporated into the H295R cells. Cells were seeded in 96-well microplates (3 x 10^4 cells/well) in complete medium. After 18 h, media was replaced with DMEM/F12 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 for the last 12 h. At the end of the pulse period, cells were frozen at -20°C and harvested in glass fiber discs by filtration. Samples were washed with 95% ethanol, dried, and counted by liquid scintillation counting.

4.7. MTT assay

This assay is based on the transformation and colorimetric quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Herein a linear relationship between cell number and signal produced was established, thus allowing the quantification of cell proliferation. In brief, cells were plated in 96-well microplates (3 x 10^4 cells/ well), and 24 h later they were treated with the indicated compounds. After 24 h, MTT was added
(final concentration 0.5 mg/ml), and 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. The color intensity was measured at 570 nm using a
multiplate ELISA reader.

4.8. Cell cycle analysis

H295R cells were seeded in 6-well microplates (1.5 x 10⁶ cell/well) in complete culture medium. After 18 h,
culture medium was replaced with DMEM/ F12 with reduced serum (1%) and incubated with HA or FMPH, both
at a concentration of 10⁻⁵ M for 24 h. After the incubation period, cells were harvested by trypsinization,
centrifuged, washed twice in PBS and fixed in PBS ice-cold ethanol (1:3). After centrifugation at 4°C, 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, 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 and CyChred analytical
software.

4.9. Tunel assay

H295R cells were cultured in 6-well microplates (1.5 x 10⁶ cell/well) in complete media. After 18 h, culture
medium was replaced with DMEM/F12 with reduced serum (1%) and cells were incubated with HA or FMPH,
both at a concentration of 10⁻⁵ M for 24 h or 72 h. After incubation, nuclear DNA fragmentation was detected by
Tunel method using the cell death detection kit (Roche Applied Science, Germany) according to the
manufacturer’s instructions. Apoptosis was analyzed by flow cytometry and data were processed with WinMDI
2.8 software.

4.10. Western Blot analysis

Total cellular protein was obtained by placing cells in lysis buffer (10 mM Tris-HCl, 1% Tritón X-100, 0.5 mM
EGTA, pH 7.4) containing a protease inhibitor cocktail (5 ug/ml leupeptin, 25 mM NaF, 25 mM sodium
orthovanadate, 400 µM PMSF, 5 µg/ml pepstatin and 5 µg/ml aprotinin), followed by 30 passages through a 1ml 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, Hercules, CA). Electrophoresis, transfer of proteins to PVDF membranes, and immunodetection of StAR, HDC, caspase-3 and β-tubulin were performed under optimized conditions.

4.11. Immunocytochemistry of HA content

Y1 and H295R cells were seeded onto a 12-mm diameter round glass coverslip, precoated with polylysine (3x10⁴ cell/coverslip) and after 3 days were washed and fixed with 4% formaldehyde for 15 min at room temperature. Cells were permeabilized for 10 min with 0.25 % TritonX-100 and 0.3 M glycine in PBS (PBST) and unspecific binding was blocked with 1% BSA in PBST for 30 min. Coverslips were incubated for 24 h with primary antibody against HA (1:100) or normal rabbit serum in PBS (negative control) overnight at 4ºC, followed by incubation with a secondary conjugated anti-rabbit antibody coupled with 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 quantification of HA immunocytochemical staining, representative cells were chosen and visualized by a 40 x 10 magnification through a Zeiss-Axiophot (Zeiss Oberkochen, Germany) with Olympus DP70 digital camera. Five hundred cells for each cell type of three independent experiments, were subjected to histogram analysis by 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.

4.12. Statistical Analysis

All experiments reported herein were repeated at least three times. If heterogeneity of variance was detected by Bartlett’s test, this was reduced by logarithmic transformation of the data before analysis. These data were then subjected to Student test or one-way ANOVA followed by Bonferroni test for multiple range comparisons. \( P \) values < 0.05 were accepted as significant.
5. Results

5.1. 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} to 10^{-5} M) for 5 or 24 h, in the absence or in the presence of 1mU/ml ACTH or 0.5 mM db-cAMP, for each cell line, respectively. Figure 1, A and B shows that HA treatment did not modify the steroid synthesis in any cell type, unstimulated or stimulated, at any HA concentration or incubation time. Figure only shows 24 h-treatment and cortisol quantification in H295R cells. At previously mentioned, 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 the steroidogenesis.

To discard a possible simultaneous activation of different receptors with antagonistic effects, the steroid production was assessed for 24 h in the presence of different specific agonists for each receptor subtype in the absence or presence of stimulus (ACTH 1mU/ml for Y1 cells or 0.5 mM dibutyryl cAMP for H295R cells). Compounds used were: FMPH as agonist HRH1, AMTH as HRH2 agonist, IMET as HRH3 agonist and VUF as HRH4 agonist, all at a concentration 10^{-5} M, that we have previously used (Medina et al. 2011; Mondillo et al. 2005; Pagotto et al. 2012). As can be seen in Figure 1, C and D, there were no differences in steroid production under any treatment condition with respect to control values.

StAR is a protein that mediates the rate-limiting step in steroid hormone biosynthesis (Stocco and Clark 1996). As we already showed that HA diminishes the levels of StAR protein in Leydig cells (Mondillo et al, 2009), we studied the effect of HA on StAR expression in adrenocortical cells.

Y1 and H295R cells were incubated for different times in the absence or presence of 10^{-5} M HA, and in the presence of 1mU/ml ACTH or 0.5 mM db-cAMP, for each cell line, respectively. The HA concentration (10^{-5} M) was the same that we previously used for treatment of MA-10 Leydig cells, showing a marked reduction in db-cAMP-stimulated StAR protein expression (Mondillo et al. 2009). In contrast with Leydig cells, but in concordance with results observed for adrenal steroidogenesis, HA did not modify the StAR protein expression at any time with respect to controls neither for Y1 cells (Figure 2A) nor for H295R (Figure 2B). The progressive
increase of StAR expression in both cell lines treated with their respective stimuli is coincident with previous
reports from our group (Piotrkowski et al. 2009) and others (Manna, et al. 2009).

5.2. Effect of HA on proliferation in Y1 and H295R adrenocortical cells

In order to evaluate the effect of HA on cellular proliferation, we performed [3H]-Thymidine incorporation assay,
Different results were obtained with both cell lines; HA did not modify Y1 cell proliferation (Figure 3A), but a
concentration-dependent inhibition was observed on H295R cell proliferation (Figure 3B) with a maximal effect
at $10^{-5}$ M HA (32.6 % inhibition respect to the control). Complete medium (5 % FCS) was used as 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 previously described, in the presence of HA, the
treatment with agonists did not modify [3H]-Thymidine incorporation in Y1 cells (Figure 3C). On the contrary, in
H295R cells, the HRH1 agonist, FMPH, inhibited the proliferation in a similar extent to that observed in the
presence of $10^{-5}$ M HA (35.7 %) (Figure 3D).

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

To exclude a non-specific toxic effect of the tested compounds over H295R cells, cell viability was evaluated
using PI staining for the detection of non-viable cells by flow cytometry. As no differences in cell viability were
found between treatments at the evaluated time (data not shown) a toxic effect of HA was discarded.

5.3. Characterization of HRH1 in H295R adrenocortical cells
Since HA inhibited H295R cells proliferation through HRH1 receptor, we considered to further characterize this receptor subtype in the cell line. A saturation binding assay was performed using [3H]-Pyrilamine as specific ligand (Figure 5B). The non linear regression fitted best a one-site model, suggesting the presence of a single class of sites for HRH1 receptor in H295R cells with a KD value of 124.4 ± 15.8 nM (95% CI = 93.52 to 155.2) and a Bmax of 4.0 ± 0.2 fmol/mg protein (95% CI = 3.7 to 4.4). The same assay for Y1 cells was done for comparison (Figure 5A). The binding to intact Y1 cells also fitted best a one-site model and a single class of sites with a KD value of 21.8 ± 8.3 nM (95% CI = 4.8 to 38.8) and a Bmax of 1.2 ± 0.2 fmol/mg protein (95% CI = 0.8 to 1.5).

### 5.4. Signaling pathway of HA anti-proliferative effect in H295R cell line

In order to evaluate the signaling pathway activated by HRH1 receptor in H295R cells, cAMP and InsPn 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. Cyclic AMP levels were not modified by neither HA nor FMPH, while both of these compounds produced a two fold increase in the total InsPn content of H295R cells above the basal level (Figure 6, A and B, respectively). Furthermore, blockage of PLC by using the specific inhibitor U73122 in the presence of FMPH prevented the decrease in [3H]-Thymidine incorporation observed with HRH1 agonist alone, whereas the U-73343, a non-functional inhibitor analog of U73122, was not able to block FMPH-induced anti-proliferative effect (Figure 6 C).

### 5.5. Effect of HA on apoptosis and cell cycle control of H295R adrenocortical cells

In order to determine whether growth inhibitory effect of HA on H295R cells affected apoptosis, 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 Figure 7A, apoptotic levels were not different between treatments and control, whereas cells treated with Doxorubicin (an apoptosis inducer) significantly increased the proportion of apoptotic cells in a concentration-dependent manner. For simplicity, Figure 7 only shows 24 h-treatment. Similar results were obtained for 72 h incubation.
In order to confirm the above results, presence of activated caspase-3 (an apoptosis marker) was evaluated by Western Blot using protein extracts from H295R cells incubated with HA or FMPH (10^{-5} M) at different times (0, 6, 18, 24, 48 and 72 h). Bands corresponding to cleaved forms of caspase-3 (corresponding to 17 and 11 molecular weight) were not detected by immunoblot at any time analyzed (Figure 7B).

The effect of HA on cell cycle progression was next examined. 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 (Figure 8A). Figure 8B shows a significant increase in the percentage of cells in G2/M phase when treated with both HA and FMPH (in % of cells: C = 4.6 ± 0.8; HA = 8.75 ± 0.9; FMPH = 10.9 ± 1.5), with a concomitant decrease in the proportion of cells in S phase (in % of cells: C = 43.0 ± 4.9; HA = 28.6 ± 1.6; FMPH = 31.2 ± 2.11).

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

In an attempt to find a possible explanation for the differential effects of HA on the proliferation of H295R versus Y1 cells, and considering the well documented correlation between HDC expression and cell proliferation in several experimental models (Falus et al. 2011), we aimed at comparing the expression levels of HDC enzyme in both cell lines by Western blot analysis. As depicted in Figure 9A, the active form of HDC enzyme (53-55 molecular weight) is expressed at significantly higher levels in Y1 compared to H295R cells. Stomach was used as positive control. Coinciding, the endogenous content of HA revealed by immunocytochemistry and quantified as described in Materials and Methods, was higher in Y1 cells (Figure 9B, upper and lower panel).

As an approach to demonstrating more directly the role of HDC and HA content on Y1 and H295R cell proliferation, we evaluated the effect of the catechin EGCG, known to potently inhibit HDC activity, as was 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 Figure 10, EGCG inhibited Y1 cell proliferation in a concentration dependent manner. EGCG concentrations higher than 4 x 10^{-5} M were toxic.
6. Discussion

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

These results agree with previous works in bovine co-cultures of adrenal medulla and cortex cells, in which it is suggested an indirect effect of HA through the HRH1 present in adrenal medulla, so inducing release of neuropeptides that would act on adrenocortical cells, regulating the secretion of cortisol (Ehrhart-Bornstein, et al. 2000; Ehrhart-Bornstein, et al. 1998; Yoshida et al. 1997). In our study, we have used adrenocortical cell lines excluding contamination with chromaffin cells. Then, according to our observations in Y1 and H295R cells, HA would not be able to directly regulate steroid synthesis of adrenocortical cells, showing that the modulatory effect observed in Leydig cells can not be extrapolated to all steroidogenic systems.

Regarding the ability of HA to regulate adrenocortical cell proliferation, results were different between tumor cell lines evaluated. Y1 murine line did not respond to treatment with HA, whereas the H295R human cells reduced growth to about 60% of control with 10^{-5} M HA. This effect was reproduced by FMPH, specific agonist for HRH1 subtype receptor.
In bovine adrenal gland, expression of HRH1 has been reported in medulla and cortex with different expression level and affinity, both being higher in medulla (Chang, et al. 1979; Yamashita, et al. 1991). Herein, the presence of a functional HRH1 was reported in the human adrenocortical carcinoma cell line H295R.

The differential effect of HA on H295R and Y1 cells proliferation could be explained, at least in part, if considering that Y1 cells showed 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 suggesting that higher HA content in Y1 cells avoids the inhibition observed in H295R cells in the presence on 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 (Hishinuma, et al. 2010; Miyoshi, et al. 2006). The lower number of HRH1 in Y1 compared to H295R cells, calculated by 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 subtype receptors, for example, HRH1 (Valencia, et al. 2001), HRH2 (Cricco et al. 2006), HRH3 (Francis et al. 2009) and by HRH4 (Meng et al. 2011).

Particularly in humans, the influence of HA on adrenocortical cells had already been suggested by Szabó et al (Szabo et al. 2009), who compared histamine-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 presented here in which the addition of HA was able to inhibit proliferation in H295R cells.

As it was previously mentioned, HA-mediated growth inhibition in H295R cells was carried out by the activation of HRH1, with an increase in InsPn, suggesting that in adrenocarcinoma cells, activation of HRH1 would be associated to the classic signaling pathway involving a phospholipase C (PLC). The reversion of the HA-antiproliferative effect in the presence of specific PLC- inhibitor U-73122 confirmed these results.
A similar HA-anti-proliferative signaling mechanism was described for prostate cancer cell line DU-145 (Valencia et al. 2001) as well as CHO cells stably transfected with HRH1, where HA activated a PLC, leading to an inhibition of proliferation through a mechanism mediated by GTPasa, Rac and e-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 without activating aldosterone production, it would be possible that HA stimulates NOS enzyme activity (via Ca\(^{2+}\)) blocking steroidogenesis as we have previously described in MA-10 Leydig cells (Mondillo et al. 2009) and it has been observed in other steroidogenic systems (Ducsay and Myers 2011). Regarding this, it has been demonstrated that NOS can inhibit L-type calcium channel (Wang et al. 2008), which is necessary for AII mediated steroidogenesis. Supposing HA induced NOS in H295R cells, the entry of calcium through the L-channel would be blocked thus preventing aldosterone synthesis, without affecting proliferation pathway. Nevertheless, an activation of other kinase signaling pathways by other HA receptors, with an antagonizing effect, can not be discarded.

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

Several proteins have been associated with the entry control to G2/M phase (Smits and Medema 2001). In this regard, in H295R cells it has been described a G2/M phase arrest induced by combinatory treatment with mitotane and ionizing radiations. These agents act by attenuating the DNA repair mechanisms and keeping high levels of cyclin B1/cdc2 complexes (Cerquetti, et al. 2010). It is likely that at least some of these events participate in the G2/M phase arrest induced by HA. Further studies must be conducted in order to confirm this hypothesis.

Currently, non-surgical treatments for human ACC are scarce and based on ionizing radiation in association with high doses of adrenalytic drugs, bringing about toxic side effects that limit its usefulness (Maluf, et al. 2011). Our results suggest 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 must be done in order to evaluate if these features could make HA a good candidate for new ACC therapies.
7 Declaration of interest

The authors have nothing to disclose. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
8. Fundings

This study was supported by grants from ANPCYT (PICT 2005-5-38281), CONICET (PIP 382) and UBA (UBACYT 2010-20020090100060 and 2013-20020120100205) to OPP.
10. Acknowledgment

The authors would like to thank Marcos Besio Moreno for skilful technical assistance.

We also thank Dr Pablo Mele and Dr Ernesto Podesta, from Dept of Human Biochemistry-School of Medicine-UBA, for the determination of aldosterone production in H295R cells.
11. References


Gazdar AF, Oie HK, Shackleton CH, Chen TR, Triche TJ, Myers CE, Chrrousos GP, Brennan MF, Stein CA & La Rocca RV 1990 Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. *Cancer Res* **50** 5488-5496.


Medina VA, Brenzoni PG, Lamas DJ, Massari N, Mondillo C, Nunez MA, Pignataro O & Rivera ES 2011 Role of histamine H4 receptor in breast cancer cell proliferation. Front Biosci (Elite Ed) 3 1042-1060.


Yasumura Y 1968 Retention of differentiated function in clonal animal cell lines, particularly hormone-secreting cultures. *Am Zool* **8** 285-305.

13. Figure legends

Figure 1: Effect of HA and its specific agonists on adrenocortical steroidogenesis. The murine cell line Y1 and the human cell line H295R, were incubated with increasing concentration of HA (A, B) or specific agonists for each HA subtype receptors, known as FMPH (HRH1), AMTH (HRH2), IMET (HRH3) and VUF (HRH4) at a concentration 10^{-5} M (C, D), for 24 h under basal and stimulated steroidogenesis. After incubation period, media were recovered and the main steroidogenic product for each cell line was quantified. (A, C) Progesterone produced by Y1 cells. (B, D) Cortisol produced by H295R cells. Bars represent the mean ± SEM of at least three independent experiments.

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 times, as described in Materials and Methods. After incubation, proteins were extracted and the expression of StAR protein was analyzed by Western Blot. Data were normalized to internal control β-tubulin. (A, B) Representative Western Blot of StAR protein in Y1 and H295R cells, respectively. (C, D) Quantitation of StAR protein levels by scanning densitometry in Y1 and H295R cells, respectively. Each bar shows the mean ± SEM of three independent experiments performed 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 concentration of HA (A, B respectively) or specific agonists for each HA subtype receptors: FMPH (H1), AMTH (H2), IMET (H3) and VUF (H4) (C,D respectively), at a concentration of 10^{-5}M, for 24 h. Cells were labeled with a pulse of [3H]-Thymidine during the last 12 h of incubation and the radioactivity incorporated into DNA was measured as described in Materials and Methods. Data are expressed as proliferation percentage respect to the control (cells incubated without HA; dashed line). Bars represent the mean ± SEM of at least three independent experiments. *, P < 0.1 vs control, **, P < 0.01 vs control; ***, P < 0.001 vs control.
Figure 4: Effects of HRH1 antagonist Pyrilamine on HA-mediated H295R cell proliferation. H295R cells were preincubated with 10^8 M Pyrilamine and after 30 minutes HA, FMPH or medium were added to the culture and incubated for 24 h. (A) Cell proliferation was measured by [3H]-Thymidine incorporation into DNA as described in Materials and Methods. (B) Cell proliferation was measured by MTT assay. Cells were incubated with 0.5mg/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 percentage respect to the control (cells incubated without HA; dashed line). Bars represent the mean ± SEM of at least three independent experiments. *, P < 0.05 vs control; **, P < 0.01 vs control.

Figure 5: Binding assay for HRH1 receptor in adrenocortical cell lines. Saturation binding assays were done in intact Y1 cells (A) and H295R cells (B) using [3H]-Pyrilamine as specific HRH1 ligand. Saturation analysis revealed a single and saturable binding site in both cell lines. Insets shows Scatchard plot of [3H]-Pyrilamine specific binding. Shown is a typical result of experiment replicated three times, with data representing mean of duplicate determinations for each cell line. Bars = SEM.

Figure 6: Signaling pathway of HA anti-proliferative effect in H295R. (A) Intracellular cAMP production. Cells were incubated for 20 minutes with 10^-5 M HA, 10^-5 M FMPH or 5 x 10^-4 M forskolin (Forsk), used as positive control. cAMP levels were measured by radioimmunoassay. (B) Total [3H]-Inositol phosphates accumulation. Cells preincubated with [3H]-Myo-inositol were treated with 10^-5 M HA, 10^-5 M FMPH or 10^-3 M NaF, used as positive control. Total [3H]-Inositol phosphates were quantified by recovered radioactivity, as described in Material and Methods. Bars represent mean ± SEM of at least three independent experiments. Different letters above the bars indicate that the groups differ significantly at least at P < 0.01. (C) Involvement of phospholipase C on H295R cell proliferation. H295R cells were incubated with the specific PLC inhibitor U-73122 or its no functional analog U-73343 in presence of FMPH for 24 h and proliferation was determined by DNA incorporation of [3H]-Thymidine during the last 12 h of incubation, as described in Materials and Methods.
Data are expressed as proliferation percentage respect to the control (cells incubated without HA; dashed line). Bars represent the mean ± SEM 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} M for 24 h, processed by Tunel reaction and analyzed by flow cytometry as described in Material and Methods. Doxorubicin was used as positive control for apoptosis. Bars represent the mean ± SEM of three independent experiments. **, P < 0.01 vs control; ***, P < 0.001 vs control (B) Evaluation of caspase-3 activation by Western blot. H295R cells were incubated with HA or FMPH (both at a concentration 10^{-5} M) at 0, 6, 18, 24, 48, and 72 h. Proteins were extracted and subjected to SDS-PAGE electrophoresis. Caspase-3 was detected using specific antibody in both forms, inactive precursor (molecular weight 32) and active subunits (molecular weight 17 and 11). Active subunits of caspase-3 were not detected even at 72 h of treatment with HA nor FMPH in H295R cells. EDS- treated MA-10 cells were used as control for anti caspase-3 antibody.

**Figure 8: Effect of HA and FMPH on H295R cell cycle progression.** H295R cells were incubated with HA or FMPH, both at 10^{-5} M for 24 h. After incubation cells were fixed, permeabilized and stained with propidium iodide as described in Material and Methods. 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 mean ± SEM of three independent experiments. *, P < 0.05 vs control.

**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 Materials and Methods. Data were normalized to internal control β-tubulin. (A, upper panel) Representative Western blot of HDC protein. Rat stomach was used as positive control. (A, lower panel) Quantitation of protein levels by scanning densitometry. Each bar shows the mean ± SEM of three independent experiments performed with
triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at 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 (AR). Each bar shows the mean ± SEM of three independent experiments, five hundred cells for each cell line were analyzed. Different letters above bars indicate that the groups differ significantly at least at P< 0.05.

Figure 10: Effect of inhibition of endogenous HDC on Y1 cell proliferation. The murine cell line Y1 was incubated with increasing concentration of EGCE, an inhibitor of HDC for 24 h. Cells were labeled with a pulse of [3H]-Thymidine during the last 12 h of incubation and the radioactivity incorporated into DNA was measured as described in Materials and Methods. Data are expressed as proliferation percentage respect to the control (cells incubated without EGCE; dashed line). Bars represent the mean ± SEM of at least three independent experiments.

*, P < 0.1 vs control; ***, P < 0.001 vs control.
Figure 1 - Y1 and H295R cell steroidogenesis
Figure 2-HA and Y1 and H295R cell StAR expression
254x190mm (96 x 96 DPI)
Figure 3-HA and Y1 and H295R cell proliferation

254x190mm (96 x 96 DPI)
Fig 4 - Pyrilamine and H295R cell proliferation
254x190mm (96 x 96 DPI)
Figure 5-Y1 and H295R cell binding
254x190mm (96 x 96 DPI)
Figure 6-cAMP and IPs in H295R cells
254x190mm (96 x 96 DPI)
Fig 7-H295R cell apoptosis
Figure 8-H295R cell cycle
254x190mm (96 x 96 DPI)
HDC activity and HA content in Y1 and H295R cells

254x190mm (96 x 96 DPI)
Proliferation of Y1 cells in presence of HDC inhibitor

254x190mm (96 x 96 DPI)