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Involvement of Nitric Oxide Synthase in the Mechanism of Histamine-Induced Inhibition of Leydig Cell Steroidogenesis via Histamine Receptor Subtypes in Sprague-Dawley Rats[1]

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ABSTRACT

This study was conducted to shed light on the so far unexplored intracellular mechanisms underlying negative modulation of Leydig cell steroidogenesis by histamine (HA). Using the MA-10 cell line and highly purified rat Leydig cells as experimental models, we examined the effect of the amine on biochemical steps known to be modulated by HA or involved in LH/hCG action. In agreement with previous findings, HA at 10 μM showed a potent inhibitory effect on hCG-stimulated steroid synthesis, regardless of the gonadotropin concentration used. Moreover, HA decreased not only LH/hCG-induced cAMP production but also steroid synthesis stimulated by the permeable cAMP analog dibutyryl cAMP (db-cAMP). Considering the post-cAMP sites of HA action, it is shown herein that HA markedly inhibited db-cAMP-stimulated steroidogenic acute regulatory (STAR) protein expression, as well as steps catalyzed by P450-dependent enzymes, mainly the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage enzyme (CYP11A). The antisteroidogenic action of HA was blocked by addition of the phospholipase C (PLC) inhibitor U73122, and HA significantly augmented inositol triphosphate (IP_3) production, suggesting a major role for the PLC/IP_3 pathway in HA-induced inhibition of Leydig cell function. Finally, HA increased nitric oxide synthase (NOS) activity, and the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) markedly attenuated the effect of the amine on steroid synthesis. On the basis of our findings, HA antagonizes the gonadotropin action in Leydig cells at steps before and after cAMP formation. NOS activation is the main intracellular mechanism by which HA exerts its antisteroidogenic effects.

histamine, Leydig cells, nitric oxide, testis, testosterone

INTRODUCTION

Histamine (HA) is a monoamine neurotransmitter synthesized exclusively by histidine decarboxylase (HDC) in most mammalian tissues. It has a role in the pathology and physiology of diverse organs [1], including the male and female reproductive systems. As regards the former, HA has been proven to be synthesized in the testis of several species [2, 3]. This locally produced HA would then exert effects on various testicular cell types, considering the recent studies [3–6] indicating expression of HA receptor subtypes 1 and 2 (HRH1 and HRH2, respectively) in germinal and peritubular cells, as well as macrophages and Leydig cells. In this respect, HA was shown to stimulate steroidogenesis and to potentiate the effects of LH in testicular parenchyma of the golden hamster [7]. Recently, our group extended those observations, reporting a dual concentration-dependent effect of the amine on steroid biosynthesis in MA-10 murine Leydig cells and in purified rat Leydig cells [6]. These results revealed a novel unreported biological activity of HA in the testis, namely, the negative modulation of steroid synthesis via HRH1. So far, the intracellular mechanisms responsible for this inhibitory action have not been fully characterized, to our knowledge.

Nitric oxide (NO) is a diffusible and short-lived free radical gas known to have a diverse range of cellular targets [8, 9]. It is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms. Neuronal NOS (nNOS or NOS I, official symbol NOS1) and endothelial NOS (eNOS or NOS III, official symbol NOS3) are responsible for the continuous basal release of NO, and their enzymatic activities are regulated by changes in intracellular concentrations of free Ca^{2+} [10, 11]. A third isoform is an inducible calcium-independent form (iNOS or NOS II, official symbol NOS2) that is regulated at the transcriptional level. Its expression can be induced in many cell types by cytokines and lipopolysaccharide (LPS) [12, 13]. Among its numerous and diverse biological actions, NO has been shown to regulate several functions within the male reproductive system under physiological and pathological conditions [14]. With particular regard to the testis, previous results from our laboratory showed that exogenously supplied NO potently inhibits LH/hCG-induced steroidogenesis in MA-10 cells and in rat Leydig cells; specifically, NO was demonstrated to directly affect cytochrome P450-dependent enzymes involved in the steroidogenic pathway, mainly cholesterol side-chain cleavage enzyme (CYP11A) [15]. In agreement, more recent studies [16–18] indicate that LPS-stimulated testicular macrophages can negatively modulate testosterone production by rat and mouse Leydig cells through paracrine NO signaling. There is also evidence to suggest that Leydig cells have the capacity to generate NO. In this respect, expression of neuronal, endothelial, and/or inducible NOS isoforms has been reported in human, rodent, and pig Leydig cells [19–23], and NO production has been detected by means of biochemical

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quantitative methods [21]. Further studies [24, 25] revealed the presence of a testis-specific variant of NOS1 (TnNOS) in human and mouse Leydig cells, in which it is encoded by the activity of a specific gene promoter. Of special interest, although NO can have a role as intracellular mediator of HA actions in various nonsteroidogenic cell types [26–28], the possibility that a similar situation might exist in Leydig cells has not been explored, to our knowledge.

In the present study, we aimed to identify the intracellular events underlying inhibition of Leydig cell steroidogenesis by HA and, in doing so, to examine the possible involvement of endogenously produced NO in the mechanism of action of the amine. Taken collectively, our results reveal multiple sites of HA action in the inhibition of Leydig cell steroidogenesis and indicate that NOS activation is the main intracellular mechanism by which HA exerts its antisteroidogenic effects. The rising prevalence of allergic diseases and the subsequent increase in the use of anti-HA medication underline the significance of this study.

MATERIALS AND METHODS

Materials

Purified hCG (CR-127, 14 900 IU/mg) was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH) (Bethesda, MD). Sodium iodide I 125 (specific activity, 600 Ci/mmol) and [^{3}H]-inositol (specific activity, 25 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). [^{3}H]-L-arginine (specific activity, 49 Ci/mmol) was purchased from Amersham International (Buckinghamshire, England). Histamine dihydrochloride, dibutyryl cAMP (db-cAMP), 2′-O-monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cAMP), 22R-hydroxycholesterol (22R-OH-Ch), pregnenolone (P_5), N^G-nitro-L-arginine methyl ester (L-NAME), protease inhibitors leupeptin and PMSF, and phospholipase C (PLC) inhibitor U73122 were purchased from Sigma Chemical Co (St. Louis, MO). Collagenase was purchased from Worthington (Freehold, NJ). Cell culture supplies and plasticware were obtained from Gibco-BRL (Gaithersburg, MD) and Corning (Corning, NY), respectively. The resins AG-I-X8 (formate form) and AG-50W-X8 (hydrogen form) were from Bio-Rad Laboratories, Inc. (Hercules, CA). The TME-cAMP was radiolabeled with $Na^{125}I$ in our laboratory by the method of chloramine T (specific activity, 600 Ci/mmol) previously described by Piroli et al. [29]. The specific antibody for cAMP was provided by Dr. A.F. Parlow (National Hormone and Pituitary Program of the NIDDK). Antibodies for progesterone and testosterone were a gift from Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins). Anti-β-tubulin monoclonal antibody E7 was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Dr. Douglas M. Stocco (Texas Tech University Health Science Center, Lubbock) and Dr. Walter L. Miller (University of California, San Francisco) generously provided steroidogenic acute regulatory (STAR) protein antibody. Anti-CYP11A antibody was kindly donated by Dr. Marta Tesone (Institute of Biology and Experimental Medicine-CONICET, Buenos Aires, Argentina). Other reagents used were of the best grade available and were obtained from commonly used suppliers.

Cellular Culture of MA-10 Leydig Cells

The MA-10 line (kindly provided by Mario Ascoli, University of Iowa, Iowa City) is a clonal strain of Leydig tumor cells that secretes progesterone rather than testosterone as a major steroid and provides a suitable model system for the study of gonadotropin actions and regulation of differentiated functions of Leydig cells. The origin and handling of MA-10 cells have been described [30, 31]. Cells were plated in 24 × 16-mm-well plates on Day 0 at a density of 1.25×10^5 cells/well and in a total volume of 1 ml of growth medium (Waymouth MB752/1 [Sigma-Aldrich Corp., St. Louis, MO], modified to contain 1.1 g/l of $NaHCO_3$, 20 mM Hepes, 50 μg/ml of gentamycin, and 15% horse serum, pH 7.4). The cells were maintained in a humidified atmosphere containing 5% Co_2 and were used on Day 3. At this time, the cell density was approximately 5×10^5 cells/well. On this day, the cells were washed twice with 1 ml of serum-free medium supplemented with 1 mg/ml of BSA (37°C, assay medium). Incubations were performed in a total volume of 0.5 ml of assay medium at 37°C with the corresponding additions as shown in each figure herein. After 5 h, media were removed, and progesterone was measured by RIA as previously described [32]. The intraassay and interassay variations were 8.0% and 14.2%, respectively. In experiments assessing the effect of PLC inhibitor U73122 or NOS inhibitor L-NAME on HA antisteroidogenic actions, cells were incubated with the specific inhibitor for 30 min before HA treatment for an additional 4.5 h. All experiments were performed in triplicate with at least three different cell line batches or passage numbers of cells, and similar results were obtained each time.

Preparation of Rat Leydig Cells

For all experiments, Leydig cells were isolated from a pool of 16 testes obtained from eight adult Sprague-Dawley rats (60 days old, 200–250 g, Charles River descendants, Animal Care Laboratory, Institute of Biology and Experimental Medicine) as previously described [6, 33, 34]. Animals were housed in groups in an air-conditioned room with lights-on from 0700 to 1900 h. They were given free access to laboratory chow and tap water. Animals were killed by decapitation according to protocols for animal use approved by the institutional animal care and use committee (Institute of Biology and Experimental Medicine–CONICET), which follows NIH guidelines.

Briefly, testes were decapsulated and dispersed by shaking (20 min, 80 cycles/min, 34°C) in 50-ml polypropylene tubes (Falcon, Cowley, England) containing 0.05% collagenase in medium 199 (M199) supplemented with 0.1% BSA (1 ml/testis). The incubations with collagenase were terminated by 1:5 dilution using M199 supplemented with 0.1% BSA. The tubes were capped and inverted several times. The seminiferous tubules were allowed to settle for 1 min, and the supernates were collected by aspiration using a pipette. The interstitial cells were filtered through Nitex monofilament (BD Biosciences Labware, Beford, MA). The tubes containing the settled seminiferous tubules were refilled with M199 supplemented with 0.1% BSA, and the same procedure was repeated. After filtering, the cells were pelleted by centrifugation at 800 × *g* for 5 min and washed twice. These interstitial cell pellets were then resuspended in 5 ml of a 1.7 mM Tris, 140 mM NH_4Cl solution, pH 7.2, as previously described [6] and incubated for 10 min at 37°C. Incubation was terminated by dilution with M199 and followed by centrifugation. This procedure eliminates any interference due to the presence of red blood cells and does not affect the cell response to gonadotropin stimulation. The cell pellets were then resuspended in 10 ml of M199–0.1% BSA. Testicular macrophages were separated by differential attachment to plastic culture plates (34°C, 15 min). The plates were washed five times with PBS to remove unattached cells. These were centrifuged at 800 × *g* for 5 min. The cell pellets were resuspended in M199–0.1% BSA and were purified by fractionation on a three-layer Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient (26%, 34%, and 60%), which was centrifuged at 800 × *g* for 30 min. The purity of Leydig cells obtained was greater than 87% as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity [35]. Less than 1.7% and 1.2% of the contaminating cells in the Leydig cell preparations were positive macrophages for ED-1 and ED-2 antigens, respectively [36]. The remaining cell types had the morphology of peritubular cells or endothelial cells. As regards mast cells, these are normally located in the testicular capsule, which is removed at the beginning of the procedure.

The pellets, enriched in Leydig cells, were gently resuspended in M199 containing 1.2 g/l of $NaHCO_3$, 20 mM Hepes, 0.1 mM 1-methyl-3-isobutylxanthine, and 0.5% BSA. Incubations were performed in plastic tubes in a volume of 500 μl (5×10^5 cells/tube) at 34°C with shaking for 5 h. The samples were then centrifuged at 800 × *g* for 5 min, and the supernatants were used to determine testosterone level by RIA [6]. The intraassay and interassay variations were 7.3% and 13.2%, respectively.

Dose-response experiments were performed to determine the optimal concentration of hCG to stimulate the cells. Testosterone production was maximal in the presence of 1 ng/ml of hCG. Therefore, all subsequent experiments in rat Leydig cells were performed using this hCG concentration. In experiments assessing the effect of NOS inhibitor L-NAME on HA antisteroidogenic actions, cells were incubated with L-NAME for 30 min before HA treatment for an additional 4.5 h. All experiments were repeated with at least three different preparations of Leydig cells obtained from different sets of animals. In each experiment, values for a given treatment group were obtained using triplicate tubes.

Determination of Intracellular cAMP in MA-10 Leydig Cells

The method for the determination of intracellular cAMP in the MA-10 cell line has been previously published [6, 37]. After a 20-min incubation of the cells in the presence of the corresponding additions, the plates were placed on ice, and the medium was aspirated. Next, 0.5 ml of cold distilled water was added to each well, and the cells were scraped and disrupted by ultrasonic oscillation. The samples were heated for three periods of 1 min in boiling water to destroy endogenous protein kinase. After centrifugation in an Eppendorf

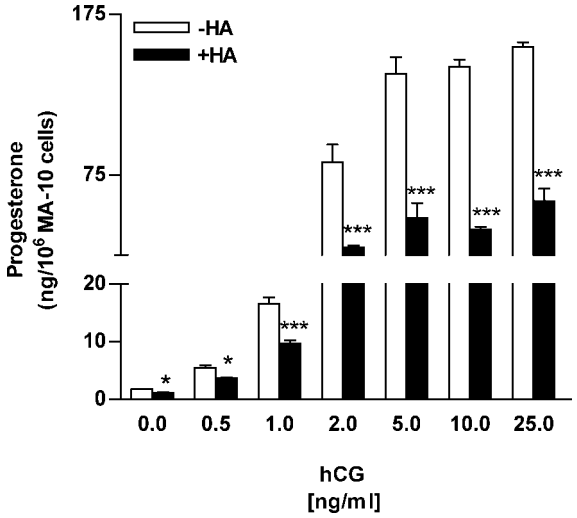

FIG. 1. Inhibitory effect of 10 μM HA on LH/hCG-stimulated progesterone production in MA-10 cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. * $P < 0.05$ vs. same treatment in the absence of HA; *** $P < 0.001$ vs. same treatment in the absence of HA.

microfuge (Brinkmann Instruments, Westbury, NY) for 3 min, samples were diluted using 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and assayed by RIA using the method described previously [15]. The interassay and intraassay variations of coefficients were lower than 10%.

Determination of [^{3}H]-Inositol Phosphates in MA-10 Cells

Inositol phosphates (IPs) were measured as described previously [6, 38]. Cells were incubated in assay medium A (Waymouth MB752/1 containing 1.1 g/l of $NaHCO_3$, 20 mM Hepes, and 1 mg/ml of albumin, pH 7.4, 37°C) with 4 to 5 μCi/ml of [^{3}H]-inositol for 24 h before the experiment. At the end of the labeling period, the cells were washed five times with 2-ml aliquots of assay medium B (Waymouth MB752/1 without $NaHCO_3$ but containing 20 mM Hepes and 1 mg/ml of albumin, pH 7.4, 37°C). Cells were then preincubated for 15 min in 2 ml of assay medium B (37°C) containing 20 mM LiCl. At the end of this period, 10 μM HA or 10 mM NaF was added. After a 30-min incubation, the wells were placed on ice, and the medium was quickly aspirated and replaced with 0.5 ml of ice-cold 0.5 M $HClO_4$. The cells were scraped with a rubber policeman and transferred to tubes. The wells were then washed with 0.7 ml of ice-old $HClO_4$, and this wash was combined with the previous extract. After a 30-min incubation on ice, the extracts were centrifuged. Collected supernatants were neutralized by the addition of 0.72 M KOH/0.6 M $KHCO_3$. The precipitated $KClO_4$ was removed by centrifugation, and supernatants were mixed with 0.5 ml of 100 mM inositol. The volume was adjusted to 5 ml with H_2O, and supernatants were chromatographed on 0.5 × 3.0-cm Dowex (Bio-Rad Laboratories, Inc.) columns (formate form). The columns had been prewashed with 10 ml of 10 mM inositol. After adding the samples, the columns were sequentially washed with 15 ml of 10 mM inositol (to wash residual [^{3}H]-inositol), 5 ml of 5 mM sodium borate/60 mM sodium formate (to elute glycerophosphoinositol), 5 ml of 0.1 M formic acid/0.2 M ammonium formate (to elute inositol monophosphate, IP_1), 5 ml of 0.1 M formic acid/0.4 M ammonium formate (to elute inositol bisphosphate, IP_2), and 5 ml of 0.1 M formic acid/1.0 M ammonium formate (to elute inositol trisphosphate, IP_3). Eluted fractions were transferred to vials containing scintillation cocktail (Optiphase Hisafe III scintillation liquid; Wallac, Gaithersburg, MD), and radioactivity was determined by liquid scintillation counting (Tri-carb 1600TR; Packard, Meriden, CT). Results are expressed as the ratio obtained when [^{3}H]-IP_1, [^{3}H]-IP_2, or [^{3}H]-IP_3 activity was normalized to total [^{3}H]-inositol recovered from the initial water wash of the columns corresponding to the intracellular [^{3}H]-inositol pool.

Preparation of MA-10 Cell Postmitochondrial Fractions and Measurement of NOS Activity

MA-10 cells were washed five times with ice-cold PBS and then scraped and resuspended in 0.5 ml/plate of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 10 μg/μl of leupeptin, 1 mM dithiotreitol (DTT), 5 mM NaF, 1 mM PMSF, and 50 mM sodium orthovanadate (homogenization buffer). Cell suspensions were then forced through a syringe using a 21-gauge needle 60 times and centrifuged at 900 × g for 10 min at 4°C. Supernatants were collected in Eppendorf tubes and centrifuged at 9000 × g for 15 min at 4°C. NOS activity was subsequently determined by monitoring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as previously described [39]. In each assay, 70 μl of MA-10 cell extract (postmitochondrial fraction) was added to 70 μl of buffer containing 50 mM Tris-HCl, pH 7.4, 10 μg/μl of leupeptin, 1 mM DTT, 5 mM NaF, 1 mM PMSF, 50 mM sodium orthovanadate, 1 mM NADPH, 1 mM $CaCl_2$ and 90 nM [^{3}H]-L-arginine (400 000 cpm). After incubation for 30 min at 37°C, samples were applied to previously equilibrated columns of 1 ml of Dowex AG-50W-X8 resin (100–200 mesh, H^+ form; Bio-Rad Laboratories, Inc.). [^{3}H]-L-citrulline was eluted with 3 ml of distilled water and then quantified in the Tri-carb 1600TR liquid scintillation counter. NOS activity is indicated in picomoles of [^{3}H]-L-citrulline formed per milligram of protein per 30 min.

Western Blot Analysis and Immunodetection of Proteins

After a 4-h incubation in the presence of the corresponding additions, total cellular protein was obtained by placing MA-10 cells in lysis buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1 M EDTA) containing a protease inhibitor cocktail (10 μg/μl of leupeptin, 5 mM NaF, 2.5 mM PMSF, and 50 mM sodium orthovanadate), followed by brief sonication. Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) for total protein. Equal amounts of protein per sample (30–40 μg) were loaded onto a 15% (w/v) SDS-polyacrylamide gel (Mini Protean III System; Bio-Rad Laboratories, Inc.). Electrophoresis, transfer of proteins to polyvinylidene fluoride membranes, and immunodetection of STAR and CYP11A were performed under optimized conditions [40–42]. The intensity of immunospecific bands was quantified using ImageJ software (NIH, Bethesda, MD) [43, 44]. To correct for equal loading and blotting, all blots were reprobed with anti-β-tubulin antibody.

Statistical Analysis

All experiments performed herein were repeated at least three times, and the data were pooled. If heterogeneity of variance was detected by Bartlett test, this was reduced by logarithmic transformation of the data before analysis. These data were then subjected to one-way ANOVA followed by Newman-Keuls test for multiple-range comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory Effect of HA on hCG-Stimulated MA-10 Leydig Cell Progesterone Production

Our previous results demonstrated that HA can exert a noncytotoxic inhibitory effect on steroidogenesis in MA-10 cells under basal conditions and after acute stimulation with a maximally efficient hCG concentration (10 ng/ml, 5 h) [6]. To further characterize the effect of HA on hCG-stimulated progesterone production, MA-10 cells were incubated with increasing hCG concentrations (0.5–25 ng/ml) in the absence or presence of 10 μM HA for 5 h. As shown in Figure 1, the inhibitory action of HA was observed at all hCG concentrations tested, with maximum inhibition observed in the presence of hCG concentrations of 5 ng/ml or higher (70% inhibition, on average). The HA affected the maximal steroidogenic capacity of the cells but not the median effective concentration of hCG (1.8 ng/ml) required to stimulate progesterone synthesis.

Involvement of PLC Activation and IP_3 Production in HA-Mediated Inhibition of Progesterone Synthesis in MA-10 Cells

We demonstrated that reduction of steroid levels by HA at 10 μM would be solely mediated via HRH1 activation. It is well known that HRH1 couples to PLC via the GTP-binding protein Gq in a wide variety of tissues [45]. Therefore, it was of

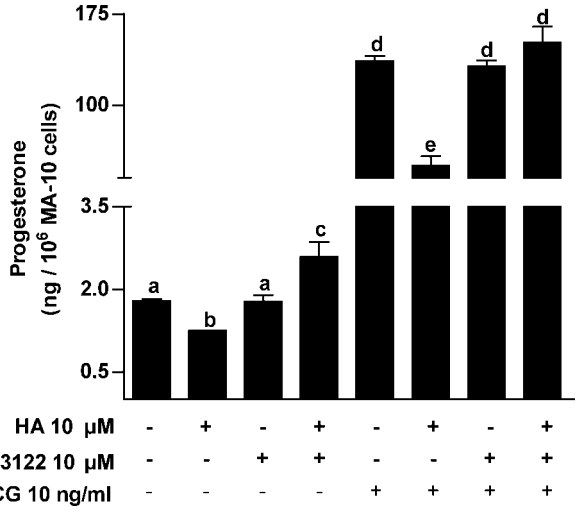

FIG. 2. Effect of the PLC inhibitor U73122 on 10 μM HA modulation of basal and hCG-stimulated progesterone production in MA-10 cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

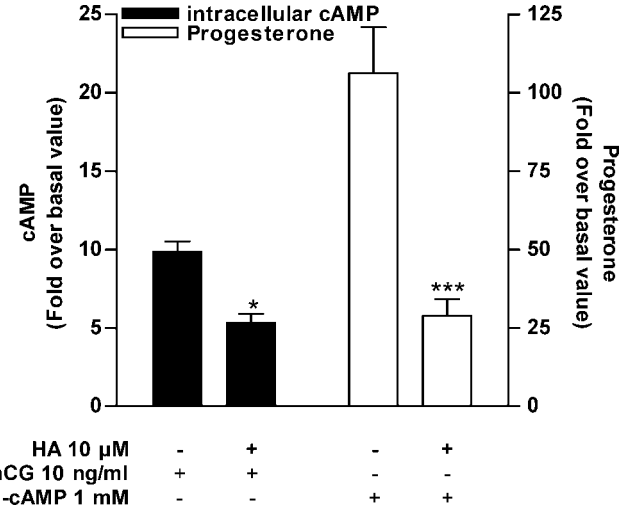

FIG. 3. Effects of 10 μM HA on hCG-stimulated cAMP production (black bars) and db-cAMP-stimulated progesterone biosynthesis (white bars) in MA-10 cells. Intracellular cAMP levels were measured as described in *Materials and Methods*. Basal cAMP levels were a mean ± SEM of 1.87 ± 0.16 pmol cAMP/10^6 cells, n = 3. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. * $P <$ 0.05 vs. hCG alone. Basal progesterone levels were a mean ± SEM of 1.42 ± 0.20 ng/10^6 cells, n = 3. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. *** $P <$ 0.001 vs. db-cAMP alone.

interest to evaluate the involvement of PLC in HA-induced inhibition of steroidogenesis. MA-10 cells were incubated with 10 μM HA in the absence or presence of the PLC inhibitor U73122 (10 μM) with or without hCG (10 ng/ml) for 5 h at 37°C. Under basal and hCG-stimulated conditions, U73122 completely reversed the inhibitory effect of HA on progesterone synthesis (Fig. 2). No detectable changes in progesterone levels were observed for U73122 alone (data not shown). We also studied whether HA could modulate accumulation of IPs. MA-10 cells were incubated with or without 10 μM HA for 30 min at 37°C. Sodium fluoride (10 mM) was used as a positive control. As shown in Table 1, 10 μM HA elicited a significant augmentation of IP_1, IP_2, and IP_3 levels in MA-10 cells.

Sites of Action of HA on Steroidogenesis in MA-10 Cells

To characterize the site(s) of HA action in MA-10 cells, we examined the effect of the amine on biochemical steps known to be modulated by HA or involved in LH/hCG action. Considering that cAMP is the main second messenger in the LH/hCG signaling pathway, we first evaluated the capacity of HA to negatively regulate cAMP production. MA-10 cells were incubated with or without 10 μM HA in the absence or presence of 10 ng/ml of hCG for 20 min at 37°C. As shown in Figure 3 (black bars), HA induced a significant decrease in LH/hCG-stimulated cAMP production (46% inhibition). Effects on basal cAMP levels were nondetectable. We then studied the effect of HA on progesterone synthesis stimulated by db-cAMP, a membrane-permeable analog of cAMP. MA-10 cells were incubated with db-cAMP (1 mM) in the absence or presence of 10 μM HA for 5 h at 37°C. The HA potently inhibited progesterone production stimulated by the nucleotide to a similar extent (73% inhibition) as that observed with the hCG (Fig. 3, white bars).

To further identify the post-cAMP site(s) of HA action, we examined the effect of the amine on the expression of the STAR protein, which mediates the rate-limiting step in steroid hormone biosynthesis (i.e., the delivery of cholesterol from the outer to the inner mitochondrial membrane) [46]. As shown in Figure 4, treatment of MA-10 cells with HA caused a marked reduction in db-cAMP-stimulated STAR protein expression. We subsequently incubated MA-10 cells with two different steroid substrates, namely, 22R-OH-Ch, a cholesterol substrate derivative that does not need an assisted process to be delivered to the inner mitochondrial membrane, and P_5. As shown in Figure 5, HA exerted a significant inhibitory effect on progesterone synthesis in the presence of 22R-OH-Ch (75% inhibition), but such effect was no longer observed in the presence of P_5. Considering that CYP11A catalyzes the conversion of cholesterol to P_5, we also evaluated the effect of HA treatment on the expression of this enzyme. Figure 6 shows that HA did not modify db-cAMP-induced CYP11A expression.

Effect of the NOS Inhibitor L-NAME on HA-Induced Inhibition of Progesterone Synthesis in MA-10 Cells

To examine the possibility that NO may be involved in HA-induced inhibition of steroidogenesis in MA-10 cells, L-NAME, a specific inhibitor of NOS activity, was tested for

TABLE 1. Effect of 10 μM HA on [^{3}H]-inositol phosphates accumulation in MA-10 cells.

Treatment	([^{3}H]IP_1/[^{3}H]-Inositol)×10^3	([^{3}H]IP_2/[^{3}H]-Inositol)×10^3	([^{3}H]IP_3/[^{3}H]-Inositol)×10^3
Control	36.04 ± 0.04[a]	8.09 ± 0.15[a]	6.68 ± 0.28[a]
NaF (10 mM)	307.19 ± 29.24[b]	67.29 ± 1.67[b]	113.59 ± 3.63[b]
HA (10 μM)	75.58 ± 2.41[c]	20.26 ± 0.36[c]	13.53 ± 1.02[c]

[a-c]Different letters in each column indicate that the groups differ significantly at least at $P < 0.05$.

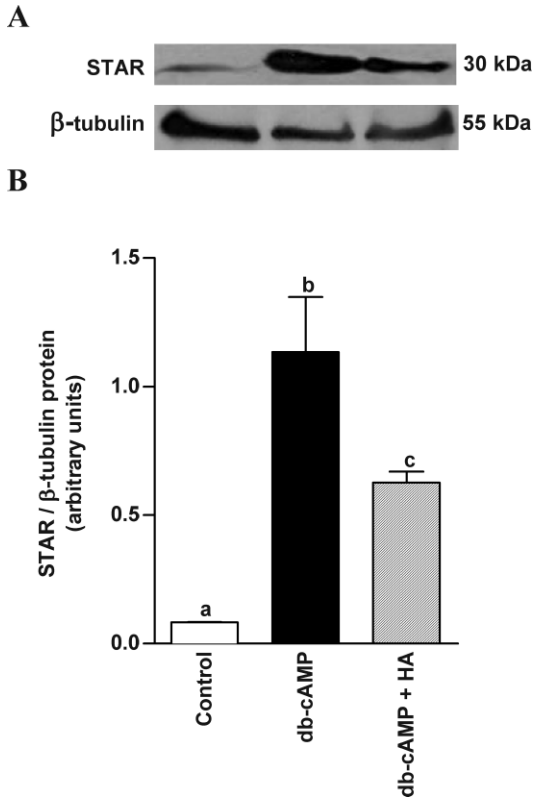

FIG. 4. Effect of 10 μM HA on STAR protein levels. After treatments, MA-10 cells were lysed and subjected to Western blot analysis as described in *Materials and Methods*. Data were normalized to the 55-kDa internal control β-tubulin. **A**) Representative Western blot of STAR protein. **B**) Quantitation 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 $P < 0.05$.

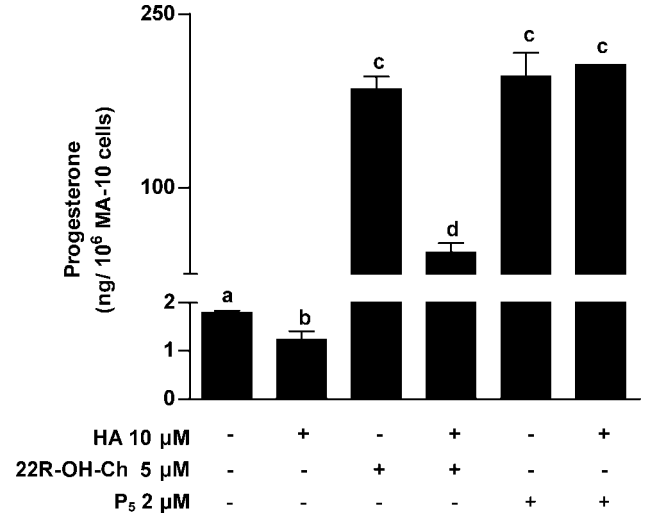

FIG. 5. Effects of 10 μM HA on progesterone formation stimulated by 22R-OH-Ch and P_5 in MA-10 cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

its ability to reverse the effects of the amine. MA-10 cells were incubated with L-NAME (1 mM), in the presence of 10 μM HA with or without hCG (10 ng/ml) for 5 h at 37°C. Figure 7 shows that L-NAME completely reversed HA-induced inhibition of basal progesterone production and potently attenuated inhibition in the presence of hCG.

Stimulatory Effect of HA on NOS Activity in MA-10 Cells

To further confirm the participation of NO in the mechanism of action of HA in MA-10 cells, we adopted a more direct approach by studying whether the amine can activate NOS in such cells. Incubations were performed in the absence or presence of 10 μM HA for 5 h at 37°C. Rat brain homogenate was used as a positive control. As expected, HA significantly increased NOS activity in MA-10 cells (2.5-fold over basal value) (Fig. 8). Clearly, these results complement our observations noted in the presence of L-NAME.

Sites of Action of HA on Steroidogenesis in Purified Rat Leydig Cells

To show that the inhibitory effect of HA on steroidogenesis was not exclusive for the MA-10 cell line, we studied the effect of 10 μM HA on testosterone biosynthesis in a suspension of highly purified normal rat Leydig cells. Figure 9 shows that HA significantly reduced, in a comparable range (about 47% decrease), testosterone synthesis stimulated with LH/hCG or db-cAMP. Effects on cAMP levels were nondetectable under basal conditions, but a 30% inhibition of hCG-induced cAMP formation was observed (data not shown). Taken collectively, these results indicate that the antigonadotropic action of HA is predominantly exerted at a step (or steps) located beyond cAMP formation, as observed for MA-10 cells. The inhibitory effect of HA on testosterone synthesis was also observed in the presence of 22R-OH-Ch and P_5 (45% and 30% inhibition, respectively) (Fig. 10).

Effect of the NOS Inhibitor L-NAME on HA-Induced Inhibition of Testosterone Production in Rat Leydig Cells

To confirm the involvement of NOS in the mechanism of action of HA in rat Leydig cells, L-NAME was tested for its ability to reverse the effects of the amine. Rat Leydig cells were incubated with L-NAME (1 mM) in the presence of 10 μM HA with or without hCG (1 ng/ml). Figure 11 shows that L-NAME completely reversed HA-induced inhibition of basal testosterone production and potently attenuated inhibition in the presence of hCG.

DISCUSSION

Convincing evidence has accumulated in recent years that locally produced HA has a role as autocrine/paracrine modulator of testicular steroidogenesis in several experimental models in vivo and in vitro [4–7, 47]. Because anti-HA drugs target HA receptors, we believe that it is essential to increase our limited knowledge regarding HA-ergic regulation of testicular functions to avoid possible unexpected adverse effects of such substances in the testis. Specifically, the present study was conducted to shed light on the so far unexplored intracellular mechanisms underlying negative modulation of steroidogenesis by HA.

In agreement with previous findings, HA at 10 μM showed a potent inhibitory effect on hCG-stimulated steroid production in MA-10 cells and rat Leydig cells, regardless of the gonadotropin concentration used [6]. The two major routes of HA metabolism result in imidazole products, which are known to affect steroidogenesis. However, our previous findings

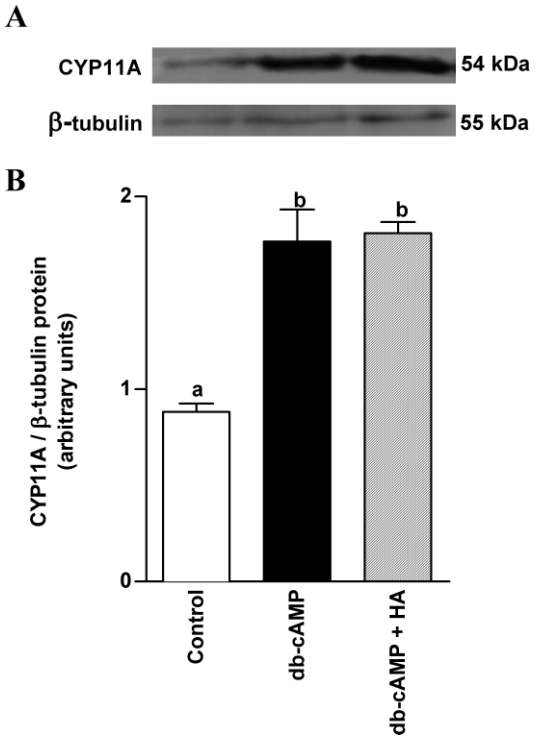

FIG. 6. Effect of 10 μM HA on CYP11A protein levels. After treatments, MA-10 cells were lysed and subjected to Western blot analysis as described in *Materials and Methods*. Data were normalized to the 55-kDa internal control β-tubulin. **A**) Representative Western blot of CYP11A protein. **B**) Quantitation 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 $P < 0.05$.

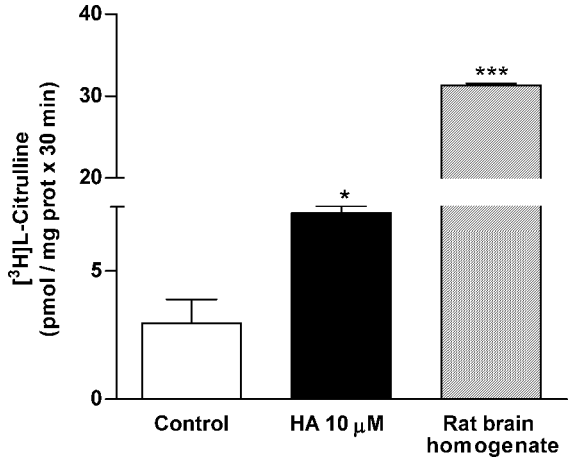

FIG. 8. Effect of 10 μM HA on NOS activity in MA-10 cells. NOS activity was determined as described in *Materials and Methods*. Rat brain homogenate was used as a positive control. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. ** $P < 0.01$ vs. control. *** $P < 0.001$ vs. control. Prot, protein.

showed complete blockage of 10 μM HA-induced inhibition of basal and stimulated steroidogenesis in the presence of the specific HRH1 antagonist pyrilamine [6]. In agreement, similar results have been recently reported in wall lizard Leydig cells [4]. These results suggest that imidazole metabolites would not be involved in HRH1-mediated inhibition of Leydig cell steroidogenesis by HA. However, we cannot completely rule out the possibility that imidazoles may exert effects on Leydig cell steroidogenesis via HRH1-independent mechanisms.

The antisteroidogenic action of HA was blocked by addition of the PLC inhibitor U73122, and HA significantly augmented IP_3 production, suggesting a major role for the PLC/IP_3 pathway in HA-induced inhibition of Leydig cell function. It is well known that HRH1 couples to PLC via the GTP-binding protein Gq in a wide variety of tissues [45]. Therefore, these results complement our former studies [5, 6] indicating that, in murine MA-10 cells and rat Leydig cells, reduction of steroid levels by HA is mediated via HRH1 activation.

The fact that steroid production was similarly and dramatically reduced in HA-treated cells stimulated with hCG or db-cAMP indicates a predominant action of HA at a biochemical step (or steps) located beyond cAMP formation. In this regard, HA provoked a marked reduction in db-cAMP-

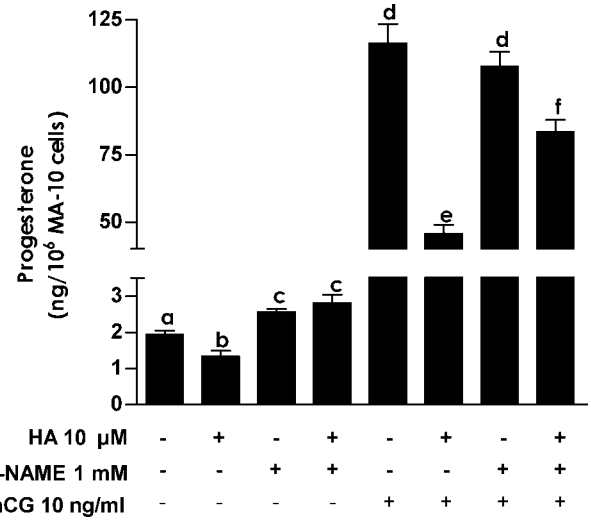

FIG. 7. Effect of the NOS inhibitor L-NAME on 10 μM HA modulation of basal and hCG-stimulated progesterone production in MA-10 cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

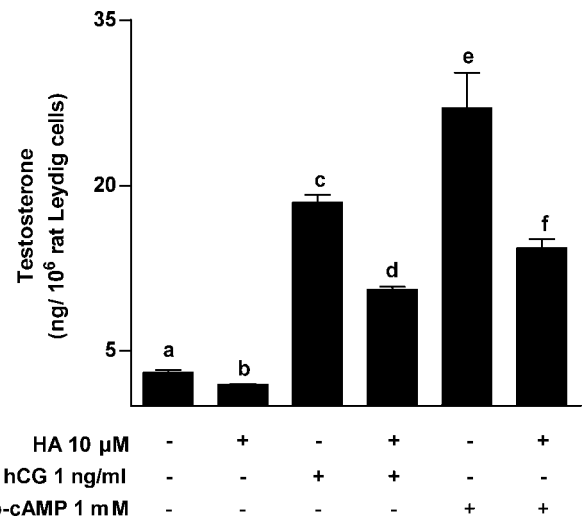

FIG. 9. Effects of 10 μM HA on testosterone production stimulated by hCG or db-cAMP in rat Leydig cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

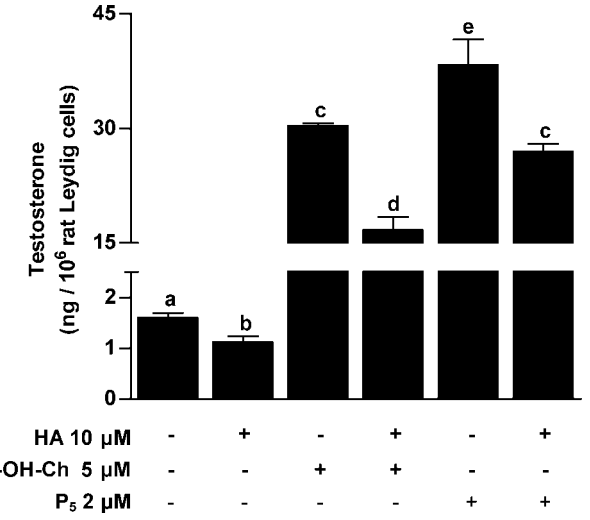

FIG. 10. Effects of 10 µM HA on testosterone formation stimulated by 22R-OH-Ch and P_5 in rat Leydig cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

stimulated STAR protein expression, implying that HA would affect intramitochondrial cholesterol transport. Furthermore, incubation of the cells with 22R-OH-Ch, which does not need an assisted process to be delivered to the inner mitochondrial membrane, did not reverse the antisteroidogenic effect of HA, suggesting the existence of at least one additional site of HA action. In the MA-10 cell model, the effect of HA was prevented when exogenously added P_5 was used as a substrate for progesterone synthesis. Considering that the truncated steroid pathway in MA-10 cells comprises two enzymatic steps, it is most likely that HA antagonizes the gonadotropin hormonal action by decreasing CYP11A activity in the inner mitochondria, while 3β-hydroxysteroid dehydrogenase activity would not be affected. Further experiments allowed us to rule out the possibility that HA may also inhibit CYP11A expression, at least after the treatment period that we evaluated.

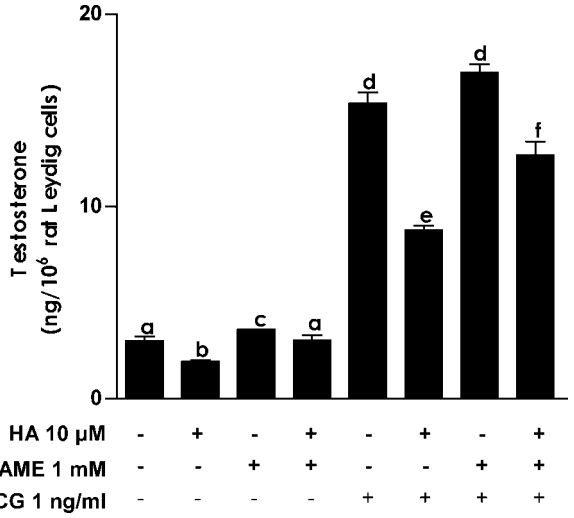

FIG. 11. Effect of the NOS inhibitor L-NAME on 10 µM HA modulation of basal and hCG-stimulated testosterone production in rat Leydig cells. Each bar shows the mean ± SEM of three independent experiments performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.

In rat Leydig cells, HA inhibited P_5-stimulated testosterone synthesis, in clear contrast to our observations in MA-10 cells. This discrepancy may be attributable to an HA-induced decrease in the activity of 17 alpha-hydroxylase/C17,20-lyase (CYP17), a P450-dependent enzyme acting beyond progesterone in the pathway leading to testosterone biosynthesis in rat Leydig cells.

NO has been extensively demonstrated to inhibit steroid biosynthesis by reversibly binding to the heme group of cytochrome P450-dependent enzymes of the steroidogenic pathway [15, 48, 49]. There is evidence in the literature suggesting a role for intracellular NO as a mediator of HA actions in various nonsteroidogenic cell types [26–28]. We speculated that a similar situation might exist in Leydig cells and conducted a series of experiments to investigate this possibility. Results presented herein show that L-NAME, a widely used NOS inhibitor, completely reversed the effect of HA on basal steroidogenesis and markedly attenuated the effect of the amine on hCG-stimulated steroid synthesis. Moreover, HA augmented NOS activity in MA-10 cells. Taken together, these findings strongly suggest the involvement of endogenously produced NO in the mechanism of HA-dependent inhibition of Leydig cell steroidogenesis. Activation of several receptor systems that lead to endothelial or neuronal NO synthesis has been shown to involve G protein-coupled signaling via PLC activation and production of IP_3 [50, 51]. NOS1 and NOS3 isoforms are expressed in Leydig cells [19, 20, 23]. Therefore, it could be speculated that a similar pathway may be responsible for the observed augmentation of NOS activity by HA. This is further supported by our findings indicating the essential role of the PLC/IP_3 pathway in HA-induced inhibition of Leydig cell function.

Our results seem to conflict with the hypothesis proposed by Weissman and coworkers [17] suggesting that Leydig cells have no endogenous NO-generating system, while testosterone production is highly sensitive to exogenous NO. However, these authors reported remarkably low NOS activity in Leydig cells, as well as a low level of expression of NOS3. We show herein that L-NAME, at the concentration used by Weissman and coworkers, almost completely counteracted the inhibitory effects of HA on steroid synthesis. Indeed, HA-induced inhibition of stimulated steroidogenesis was limited to less than 30% in cells treated with L-NAME. We also detected NOS activity in MA-10 cells in the same range as Weissman and coworkers reported for normal rat Leydig cells, and recent studies have provided clear evidence that Leydig cells not only express the NOS subtype that is specific to the male gonad but also respond to stimulation of the brain-testicular pathway by producing increasing levels of this enzyme [52]. Therefore, on the basis of these findings, we propose that Leydig cells have an NO-generating system on which testicular factors may exert a paracrine and/or autocrine regulatory effect. We demonstrate herein that HA could be one of those factors. However, we do not exclude the possibility of a paracrine effect of macrophage-related NO on Leydig cell steroidogenesis, as suggested by Weissman et al. [17] and by Hales 18]. In this regard, the existence of functional HRH1 has been recently demonstrated in testicular macrophages [4].

Of particular interest, there is evidence to suggest an intracellular NO-mediated inhibition of STAR protein expression in Leydig cells [52, 53], implying the existence of an additional site of NO action in the regulation of steroidogenesis. In view of these findings, it is tempting to speculate that NO may at least partially account for the observed inhibitory effects of HA on the expression of STAR protein, as well as the activity of CYP11A. In MA-10 cells and in rat Leydig cells,

HA provoked a significant decline in LH/hCG-stimulated cAMP production. This effect is probably not mediated by NO, considering our previous findings indicating that the gas does not modify basal or hCG-stimulated cAMP levels in MA-10 cells [15]. Therefore, it is possible that such decreased cAMP generation may exert some contribution to the observed HA-induced reduction in steroid synthesis under hormonal stimulation independent of NOS activation. If this was the case, it might help explain why L-NAME could not completely counteract the effects of the amine on the steroidogenic process. Because incubations of MA-10 cells and rat Leydig cells for subsequent cAMP determination were performed in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine, it could be speculated that HA, acting via HRH1, decreases adenylate cyclase (AC) activity. Consistent with this hypothesis, inhibition of AC selectively mediated by IP_3-evoked calcium (Ca^{2+}) release has been recently reported in A7r5 smooth muscle cells [54]. Moreover, Pereira et al. [55] have reported that Ca^{2+} decreases the rate of cAMP accumulation induced by hCG in MA-10 cells. Clearly, this hypothesis requires further investigation.

Pap et al. [47] have reported that HDC-deficient mice (homozygous for the *Hdc*^{tm1Nagy} allele, herein denoted as *Hdc*$^{-}$) show reduced testis weight and elevated testicular steroid levels compared with wild-type mice. Moreover, *Hdc*$^{-/-}$ mice show altered Leydig cell ultrastructure already at the age of 7 days, when the testes have not yet descended from the abdomen [47]. Gaytan et al. [56] have reported simultaneous proliferation and differentiation of mast cells and Leydig cells in the rat testis, suggesting the existence of dynamic interactions between the two cell types. Considering that the HA concentration in rat neonatal testes has been found to be in the micromolar range [2], it could be speculated on the basis of the observations by Pap and coworkers [47] that HA has a role as a negative modulator of testicular steroidogenesis in neonates. However, it is uncertain whether this is a direct effect of HA on fetal Leydig cells, or if other testicular cell types expressing HA receptors might be involved by paracrine actions in the modulation of steroidogenesis. In this regard, given that there are no reports (to our knowledge) on the existence of functional HA receptors in fetal Leydig cells, it is still open to debate whether these cells express such receptors or whether they do so later in development. The normal HA concentration in an adult rat testis is in the nanomolar range. However, there is evidence to indicate that it can increase significantly under stress conditions [57, 58]. This fact highlights the importance of our experiments showing how adult Leydig cells can respond to such increased HA concentration, as well as the intracellular mechanisms underlying this response. Considering that HA has also been implicated in spermatogenesis, penile erection, and sexual behavior [59–61], it seems that the amine has an integral role in the regulation of male reproductive function that deserves further investigation. Of note, studies [62, 63] have linked testicular mast cells to the pathogenesis of testicular disorders. Bearing this in mind, a potential role of HA in testicular pathology associated with infertility should also be considered.

In conclusion, results presented herein demonstrate for the first time (to our knowledge) that NOS activation is the main intracellular mechanism by which HA exerts antisteroidogenic actions in Leydig cells, suggesting a direct interaction between HA and NO to modulate Leydig cell steroidogenesis. Most important, HRH1 and NOS isoforms are found in other testicular cell types such as peritubular cells, germ cells, and macrophages [3, 4, 22, 23]. Therefore, it is possible that under specific circumstances activation of NOS and subsequent NO production comprise a general mechanism triggered by HA on its multiple testicular target cells. This mechanism may have important implications for male reproductive function under physiological and pathological conditions.

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