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# Prolonged Histamine Deficiency in Histidine Decarboxylase Gene Knockout Mice Affects Leydig Cell Function

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**ABSTRACT:** The present study focuses on histaminergic regulation of Leydig cell physiology, since limited information is available so far. To evaluate the dependency of Leydig cells on histamine (HA), we performed experiments using highly purified Leydig cells in culture, isolated from wild type (WT) and histidine decarboxylase (Hdc) gene knockout (HDC KO)—so HA-deprived—mice. HDC KO Leydig cells showed lower basal and human choriogonadotropin (hCG)-induced testosterone production compared to WT Leydig cells, presumably due to altered P450scc gene (Cyp11a1) expression levels. Moreover, in HDC KO cells, hCG did not increase basal expression levels of HA H1 and H2 receptor genes, while the hormone showed a significant inducing effect in WT cells. Based on these findings, we propose that prolonged HA deficiency in HDC KO mice affects various aspects of Leydig cell physiology, most importantly the response to hCG, providing definite evidence that HA plays a role as direct modulator of Leydig cell function and steroid synthesis in the testis. Also, the results presented herein constitute the first molecular evidence for the expression of HA H1 and H2 receptor subtypes in isolated Leydig cells.

Key words: Steroidogenesis, human choriogonadotropin.
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Histamine (HA) is a biogenic amine that plays an important role in several physiological processes. The actions of HA are mediated by 4 pharmacologically and molecularly defined receptor subtypes (H1–H4). The expression of these receptors is tissue-specific (Hill, 1990; Liu et al, 2001). In eukaryotes, HA is synthesized by a unique enzyme, L-histidine decarboxylase (HDC) (Yatsunami et al, 1994). Although mast cells are the main source of HA, many other cell types express HDC and synthesize HA themselves.

The presence of HA in animal gonads is indisputable (Nistal et al, 1984; Van Thiel et al, 1987; Gaytan et al, 1992; Almeida et al, 1996; Hibi et al, 2001). Very recent results proved the existence of a histaminergic system in the human testis as well (Albrecht et al, 2005). Most importantly, HA has been previously suggested to affect processes related to male fertility, such as gonadal development, spermatogenesis, and sexual behavior. However, the precise role of the amine in the testis is still poorly understood.

In particular, histaminergic regulation of Leydig cell physiology has not been fully investigated yet, and the limited evidence available so far seems contradictory. Mayerhofer et al (1989) demonstrated that HA can stimulate in vitro testosterone synthesis in the golden hamster; meanwhile, HA-deprived histidine decarboxylase gene (Hdc) knockout (HDC KO) mice showed increased in vivo testicular steroid production (Pap et al, 2002). To complicate the picture, HA was recently reported to exert a dual concentration-dependent effect on steroidogenesis in rat Leydig cells, as well as in the MA-10 mouse Leydig tumor cell line. H1 and H2 receptor subtypes were detected in such cells using radioligand binding assays, but no molecular evidence was shown to support these observations (Mondillo et al, 2005).

To further evaluate a direct role for testicular HA in modulation of Leydig cell physiology, we performed experiments on highly purified Leydig cells in culture, isolated from testes of HDC KO and wild type (WT) mice.

We measured testosterone levels in the incubation media and evaluated *Cyp11a1* expression in the cells using quantitative real-time polymerase chain reaction (PCR) assays. *Cyp11a1* gene (herein denoted CYP11A)

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codes for cholesterol side chain cleavage cytochrome P450 (P450scc), the main quantitative regulatory enzyme of steroid synthesis. We also performed quantitative real-time PCR assays to study expression levels of H1 and H2 receptor genes (*Hrh1* and *Hrh2*, herein denoted H1 and H2) in both experimental models, providing molecular evidence that Leydig cells are direct targets of HA action.

## Materials and Methods

### Animal Model

The experiments were conducted in accordance with accepted standards of animal care. *Hdc* targeted mice were generated as described (Ohtsu et al, 2001). Briefly, using isogenic mouse genomic DNA obtained by amplification of 129Sv-derived E14 ES cell DNA, we designed the Hdc targeting construct to replace a ~2.4-kb fragment extending from the *Spe*I site in intron 5 to the *Pst*I site in exon 9 with a PGK-*neo*$^r$ cassette. The cloned fragments were linearized and introduced into the ES cell line R1 selected by G418 and ganciclovir. Out of 6 long and accurate polymerase chain reaction (LA-PCR) positive clones aggregated with CD1 morulae, 3 were confirmed to be homologous recombinants by Southern blotting. WT and HDC KO mice were bred in our transgenic facility.

Normal rodent diet (HA content >50 μmol/g) was provided ad libitum (Charles River, Hungary). Adult male mice from multiple litters (3 to 5 months old) were used in this study. Mice were killed with ether anesthesia. Their testes were removed and kept in 4°C phosphate buffered saline buffer (PBS) until culture preparation was started. All procedures were carried out under sterile conditions.

### Preparation of Leydig Cell Culture

For each experiment, 12 to 14 testes of WT and HDC KO mice were used to obtain interstitial cells, and these pooled cells were used for subsequent Leydig cell isolation.

Testes were decapsulated and dispersed by shaking (10 minutes, 80 cycles/min, 34°C) in a tissue culture flask containing 0.025% collagenase, in Medium 199 (M199) supplemented with 0.1% BSA (1 mL/testis). The crude cell suspension was filtered twice through Nitex monofilament. Interstitial cells were pelleted by centrifugation and washed twice. The cells were then resuspended in 5 mL of a 1.7 mmol Tris, 140 mmol $NH_4Cl$ solution, pH 7.2, and incubated for 10 minutes at 37°C. This procedure eliminates red blood cells and does not affect the cell response to gonadotropin stimulation. The unfractionated interstitial cell preparation was resuspended in 10 mL M199-0.1% BSA. Testicular macrophages were separated by differential attachment to plastic culture plates (34°C, 15 minutes). Unattached cells were centrifuged, and the cell pellet was then purified by fractionation on a 5-layer Percoll density gradient (21%, 26%, 34%, 40%, and 60%). The purity of Leydig cells obtained was assessed by histochemical staining for 3-β-hydroxysteroid dehydrogenase activity (Payne et al, 1980; Del Punta et al, 1996; Mondillo et al, 2005). Isolated Leydig cells were plated in 24-well plates and maintained under a water-saturated atmosphere of 5% $CO_2$ and 95% air at 34°C in a 1:1 mixture of Ham's F-12: Dulbecco modified Eagle medium, supplemented with 15 mmol $NaHCO_3$, 20 mmol Hepes, pH 7.4, 0.1% Gentamicin, 10 μg/mL transferrin, 5 μg/mL vitamin E, and 5% fetal bovine serum (unless otherwise stated, all materials were purchased from Sigma-Aldrich Ltd, St Louis, Mo).

### Steroid Concentration Measurements

Testosterone was measured from media with ELISA kit (R and D Systems, Inc, Minneapolis, Minn) after 5 hours of incubation in the absence or presence of human choriogonadotropin (hCG) (Human Co, Gödöllő, Hungary) at concentrations ranging from 0.45 to 9 mIU/mL, as indicated in each figure legend). Results are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. $P$ less than .05 was considered significant.

### Expression of Enzymes and Receptors

Total RNA was collected from the cells after a 35-minute incubation in the absence or presence of 9 mIU/mL hCG.

### Total Cytoplasmic RNA Preparation

Briefly, samples were homogenized using TRI reagent (Sigma-Aldrich Ltd). This was followed by extraction with chloroform, precipitation with isopropanol, and final wash with alcohol. For H1 and H2 receptor studies, RNA was treated by RNase-free DNase (Promega Corp, Madison, Wis).

### Reverse Transcription Polymerase Chain Reaction

Reverse transcription (RT) of an aliquot of total cytoplasmic RNA (1 μg) to cDNA was catalyzed by multireverse transcriptase (Perkin–Elmer) in the presence of 1 mM of each dNTPs, RNase inhibitor, random hexamers, 10PCR buffer, and $MgCl_2$ (Promega) to a total volume of 40 μL. The RT mix was incubated at 42°C for 10 minutes for reverse transcription, followed by 95°C for 10 minutes to inactivate the reverse transcriptase. The cDNAs were stored at −20°C until use.

### Quantitative Real-time PCR

The assays were performed based on Assays-on-Demand Gene Expression Products Protocol (Applied Biosystems). Briefly, target cDNA samples synthesized from the RNA sample were amplified by AmpliTaq Gold DNA polymerase in the TaqMan Universal PCR Master Mix, using sequence-specific primers and TaqMan MGB probe. QRT-PCR was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif), and the associated ABI 2.0 software was used to analyze the data and determine the threshold count regarding relative quantification of gene expression experiments referring to Sequence Detection System User Bulletin #2 (P/N 4303859). The primers and probes were designed using the Assays-on-Demand Gene Expression Product list of Applied Biosystems.

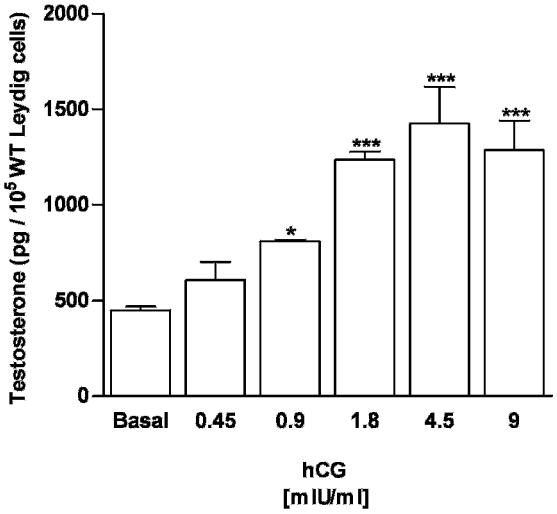


Figure 1. Dose-response curve for hCG-induced steroidogenic response in cultured WT Leydig cells. Each bar and vertical line represent the mean +/− SEM. * indicates $P < .05$ vs basal value; ***, $P < .001$ vs basal value.

Results are expressed as relative expression referred to the expression of housekeeping gene *Hprt1* (hypoxanthine-guanine phosphoribosyl transferase, herein denoted HPGRT). By using HGPRT endogenous control as an active reference we could normalize quantitation of our mRNA targets for differences in the amount of total RNA added to each reaction. Each sample was assayed in triplicate. Results are presented as the mean ± SEM. Differences between relative gene expression levels in WT and HDC KO cells were determined by Student's *t* test. *P* less than .05 was considered significant.

## Results

### *Testosterone Synthesis by Cultured Leydig Cells of WT and HDC KO Mice*

WT Leydig cells were incubated in the absence or presence of hCG (0.45 to 9 mIU/mL) for 5 hours at 34°C. As indicated in Figure 1, the hormone elicited a concentration-dependent increase in testosterone production, showing maximum effectiveness at concentrations between 1.8 and 9 mIU/mL. Based on these results, 9 mIU/mL hCG was chosen to carry out the subsequent experiments in WT and HDC KO mice. WT and HDC KO Leydig cells were incubated with or without 9 mIU/mL hCG for 5 hours, and testosterone was measured in the incubation media. HDC KO Leydig cells showed decreased basal and hCG-induced testosterone production compared to WT Leydig cells. Moreover, treatment of KO cells with hCG only elevated testosterone levels to basal WT values (Figure 2).

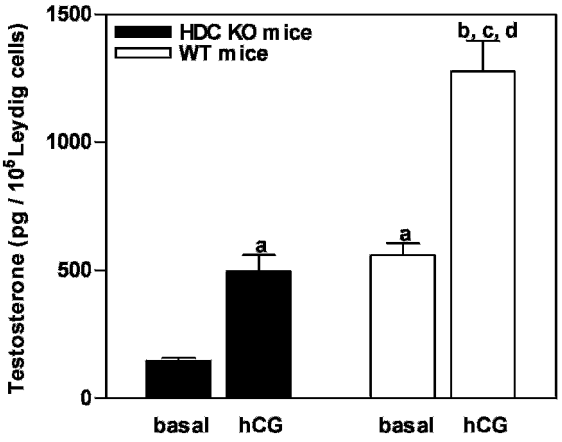


Figure 2. Testosterone levels in the medium of primary Leydig cell culture under basal conditions and after stimulation with 9 mIU/mL hCG. Both basal and hCG-induced levels are higher in the case of wild-type culture (open columns) than in the case of HDC KO culture (black columns). Each bar and vertical line represent the mean +/− SEM. **(a)** $P < .05$ vs basal value for KO Leydig cells; **(b)** $P < .001$ vs basal value for KO Leydig cells; **(c)** $P < .001$ vs hCG-induced testosterone levels for KO Leydig cells; **(d)** $P < .001$ vs hCG-induced testosterone levels for WT Leydig cells.

### *Relative Expression of CYP11A Gene*

WT and HDC KO Leydig cells were incubated with or without 9 mIU/mL hCG, and total RNA was extracted from the cells. The RNAs were reverse-transcribed, and real-time amplification was performed as described in "Materials and Methods." hCG showed no significant inducing effect on CYP11A relative expression levels in either WT or HDC KO Leydig cells. However, results indicate a small tendency for basal CYP11A relative expression rate to be lower in HDC KO Leydig cells (Figure 3).

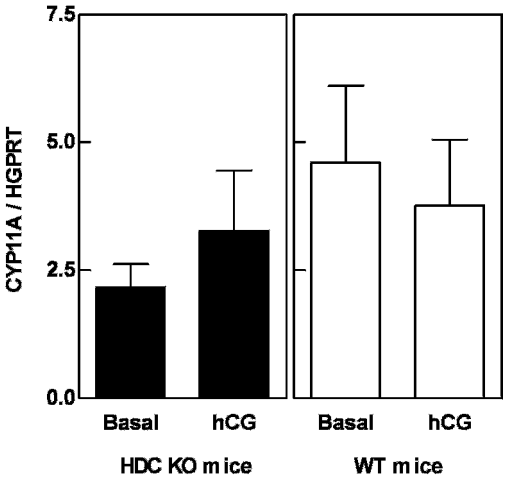


Figure 3. Relative expression of CYP11A gene in cultured HDC KO Leydig cells (black columns) and WT Leydig cells (open columns), at basal level and after 9 mIU/mL hCG induction. Each bar and vertical line represent the mean +/− SEM.

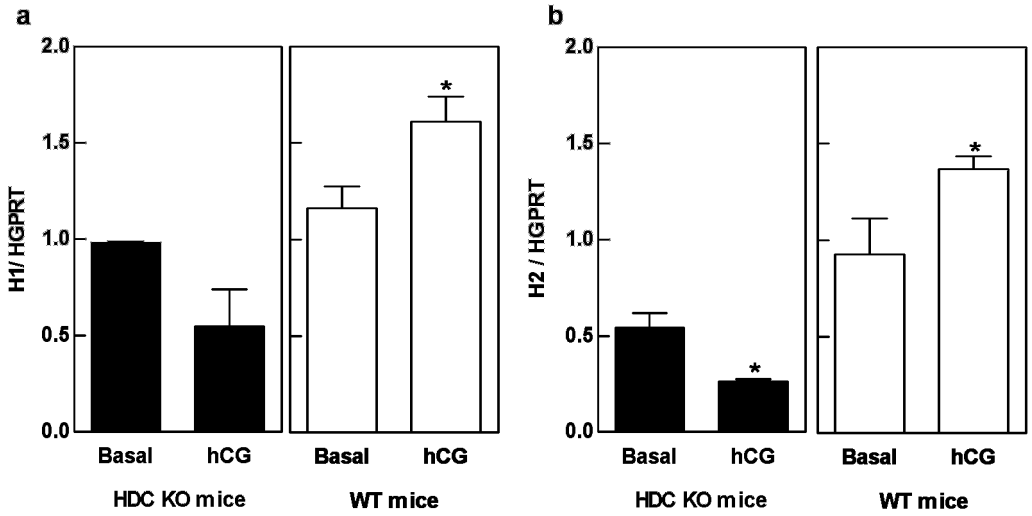


Figure 4. **(a)** Relative expression of H1 gene in cultured HDC KO Leydig cells (black columns) and WT Leydig cells (open columns), at basal level and after 9 mIU/mL hCG induction. Each bar and vertical line represent the mean +/− SEM. *, $P < .05$ vs basal stage. **(b)** Relative expression of H2 gene in cultured HDC KO Leydig cells (black columns) and WT Leydig cells (open columns), at basal level and after 9 mIU/mL hCG induction. Each bar and vertical line represent the mean +/− SEM. *, $P < .05$ vs basal stage.

### *Relative Expression of H1 and H2 Receptor Genes*

WT and HDC KO Leydig cells were incubated with or without 9 mIU/mL hCG, and total RNA was extracted from the cells. The RNAs were reverse-transcribed, and real-time amplification for the target genes was performed as described in "Materials and Methods." In WT Leydig cells, hCG treatment significantly elevated H1 relative expression levels. However, the hormone had no inducing effect in KO Leydig cells (Figure 4a). With regard to H2 relative expression rate, WT and KO Leydig cells showed an even more striking difference in their response to hCG treatment. While the hormone exerted a significant stimulatory effect in WT Leydig cells, its effect was clearly inhibitory in KO cells (Figure 4b).

## *Discussion*

The present study demonstrates experimentally that steroidogenic efficiency is significantly lower in HDC KO Leydig cells compared to WT Leydig cells. It is notable that very low serum HA content was measured in HDC KO mice, which originated exclusively from the normal diet the mice were kept on, since cells lacking functional HDC enzyme are unable to synthesize endogenous HA. Also, previous research revealed extremely low levels of HA in the reproductive organs of HDC KO mice; meanwhile, its presence was abundant in WT animals (Pap et al, 2006). Therefore, the difference in Leydig cell function found between the 2 experimental groups is clearly a consequence of the lack or presence of endogenously produced HA.

Our observations appear to conflict with previous in vivo results obtained in HDC KO mice, which indicated elevated testicular steroid levels compared to WT mice (Pap et al, 2002). However, experiments carried out using testicular homogenate may not be directly comparable to those using isolated Leydig cells, because of the presence of cell types other than Leydig cells in the in vivo case, which might exert some influence on the steroidogenic process via paracrine secretions. Considering those recent reports that indicate the presence of HA receptors in peritubular and germinal cells (Albrecht et al, 2005), such cells might also be affected by the lack of HA; more dramatically, testes of HDC KO mice lack endogenous HA from the very beginning of their embryonic development. Thus, the experiments carried out in this study—that is, using purified Leydig cells—enable a more direct determination of the effects of HA deficiency on Leydig cell function.

Our present observations also indicate that HDC KO Leydig cells cannot respond to LH/hCG stimulus as effectively as WT cells do. In this regard, even after a 5-hour induction in the presence of maximally stimulating hCG, testosterone levels synthesized by HDC KO Leydig cells reached only the levels of untreated WT cells. Given that occupation of as little as 10% of the total LH/hCG receptors in Leydig cells suffices to induce maximal steroid production (Catt et al, 1974; Barañao and Dufau, 1984), it seems quite improbable that the decreased testosterone accumulation in KO Leydig cells can be attributable to a reduction in the number of LH/hCG receptors. P450scc plays a central role in the steroidogenic pathway leading to testosterone

synthesis in Leydig cells (Nolan and Payne, 1990). The interplay between intracellular HA and different cytochrome P450 enzymes has been described in several studies (Brandes et al, 1998; LaBella et al, 2000). Therefore, we evaluated the relative expression rate of CYP11A gene in WT and HDC KO Leydig cells, under basal conditions and after hCG treatment. We found no statistically significant differences, but a tendency for CYP11A relative expression rate to be lower in HDC KO Leydig cells. Also, we cannot discard the possibility of appearing differences at post-transcriptional level or alteration of other key regulatory step(s) in the steroidogenic pathway leading to androgen synthesis, potentially before the action of P450scc enzyme.

Interestingly, Leydig cell ultrastructure is seriously altered in adult HDC KO mice, and testicular weight is significantly reduced already in 7-day-old litters, at a time when the testes have not yet descended from the abdomen. Importantly, lack of HDC does not interfere with the general morphology and distribution of GnRH neurons in these mice, which also show normal hypothalamic *Gnrh1* mRNA expression (Pap et al, 2002). Thus, although LH is the primary factor influencing morphology and function of Leydig cells (Lejeune et al, 1996), both important to maintain their steroidogenic potential, the absence of endogenously produced HA in HDC KO mice may probably result in embryonic modifications of testicular ontogenesis, leading to subnormal adult Leydig cell function. This hypothesis is reinforced by previous research indicating that testicular HA concentration is normally higher in the immature gonad than in the adult one (Zieher et al, 1971), suggesting the occurrence of important HA-dependent events during testicular development.

Very recently, we reported the existence of H1 and H2 receptors on the membrane of rat Leydig cells and MA-10 mouse Leydig tumor cells, based on binding studies (Mondillo et al, 2005). The work represents the only evidence available so far regarding the presence of HA receptors in purified Leydig cells. However, it has been argued that HA receptor subtypes cannot be distinguished solely on the basis of pharmacological studies. Also, HA-deprived HDC KO mice were previously shown to have altered expression levels of H1 and H2 receptor genes in certain tissues (Fitzsimons et al, 2001). Thus, we evaluated the expression rates of H1 and H2 receptor genes in WT and HDC KO Leydig cells, under basal conditions and after hCG treatment. In agreement with previous biochemical studies, we detected basal expression of H1 and H2 receptor genes in WT Leydig cells. Moreover, hCG showed a significant stimulatory effect. These results provide the first molecular evidence for the expression of H1 and H2 receptor subtypes in isolated Leydig cells and reveal an inducing effect of hCG on the gene expression levels of such receptor subtypes. In addition, these findings complement our recently described results, which showed that H2 receptor activation can potentiate the steroidogenic effect of hCG in rat Leydig cells and MA-10 mouse Leydig tumor cells. Also, H1 receptor activation was shown to exert a minor but significant contribution to the potentiating effect of HA (Mondillo et al, 2005).

In contrast to WT Leydig cells, hCG had no inducing effect on the relative expression rates of H1 or H2 receptor genes in KO Leydig cells. Most importantly, the hormone exerted an inhibitory effect on H2 receptor gene expression levels. Although the physiological significance of these findings requires further investigation, it seems clear that the absence of endogenously produced HA in HDC KO mice alters the way Leydig cells respond to hCG-induced regulation of gene expression levels of H1 and H2 receptors. Based on solid experimental evidence, G-protein-linked signal transduction pathways may converge and diverge at multiple levels, enabling cells to coordinate responses to diverse environmental stimuli (Brady and Limbird, 2002; Bakker et al, 2004). Both hCG and HA act through G-protein-dependent pathways. Thus, it is plausible that in the absence of HA, altered cross-talks of the transducing systems modify the response of HDC KO Leydig cells.

On the basis of the results reported herein, we suggest that prolonged HA deficiency in HDC KO mice affects various aspects of Leydig cell physiology, providing definite evidence that HA plays an important role as direct modulator of Leydig cell function and steroid synthesis in the testis.

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