INHIBITORY EFFECTS OF ACUTE AND CHRONIC TREATMENTS WITH ADRENOCORTICOTROPIN-RELEASING HORMONE ON THE LEYDIG CELLS OF THE RAT TESTIS

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ABSTRACT

The effects of acute and chronic administrations of corticotropin-releasing hormone (CRH) on the secretory activity and growth of Leydig cells of hypophysectomized/human chorionic gonadotropin (hCG)-treated rats were investigated by radioimmunoassay (RIA) and morphometry. CRH bolus intraperitoneal injection (30 or 300 pmol/kg) did not affect basal plasma testosterone concentration (PTC), but it did notably blunt the hCG-induced rise in PTC. Prolonged (7-day) subcutaneous infusion with CRH (30 or 300 pmol/kg/h) lowered basal PTC and evoked a marked decrease in the volume of testis, number of Leydig cells per testis and volumes of Leydig cells and their nuclei. Electron microscopic stereologic analysis revealed that the Leydig-cell atrophy induced by the highest dose of CRH was due to decreases in the volume of all the organelles involved in cholesterol and steroid synthesis (i.e. mitochondria, smooth endoplasmic reticulum and peroxisomes), and was associated with a striking accumulation of lipid droplets (i.e. the intracellular stores of cholesterol). Isolated atrophic Leydig cells displayed a notable reduction in both their basal and hCG-stimulated testosterone secretion. These findings indicate that CRH is able to acutely depress the hCG-enhanced secretion of testosterone *in vivo*, and, when chronically administered, to inhibit the growth and steroidogenic capacity of rat Leydig cells, without interfering with the hypophyseal release of gonadotropins.

Pro-opiomelanocortin (POMC) is produced by both Leydig cells and spermatocytes (6, 15), and corticotropin-releasing hormone (CRH) is reported to stimulate β -endorphin secretion in primary cultures of rat Leydig cells (13). β -Endorphin in turn acts as a paracrine inhibitor of Sertoli cells by blocking cyclic-AMP-mediated spermatogenic action of FSH (16). *In vitro* data also indicate that CRH is able to depress human chorionic gonadotropin (hCG)-stimulated secretory activity of rat Leydig cells (33).

To gain insight into the possible physiological role of CRH as a modulator of testis function, it seemed worthwhile to examine the *in vivo* effects of acute and chronic administrations of CRH on the secretory activity and growth of Leydig cells in rats.

Since CRH is the physiologic stimulator of the release of POMC-derived peptides by pituitary corticotropes (see 4, for review), and ACTH has been reported to enhance testosterone secretion in adult male rats (1), we employed hypophysectomized (Hx) rats treated with maintenance doses of hCG.

MATERIALS AND METHODS

Male Wistar rats (300 ± 30 g in body weight) were purchased from Charles-River (Como, Italy). A number of animals were hypophysectomized by the parapharyngeal approach, and the completeness of the operation was checked at the time of autopsy, by inspecting sella turcica under the dissecting microscope. Moreover, sellae turcicae of

randomly chosen rats were processed for light microscopy and serially cut: the histologic examination never revealed remnants of pituitary tissue. Other rats were sham-operated (control rats). On the third day after hypophysectomy, groups of rats were subcutaneously infused for 7 days (Alzet osmotic pumps Mod. 2001; Alza, Palo Alto, U.S.A.) with hCG (Serono, Rome, Italy) dissolved in 0.9% NaCl vehicle (1 IU hCG/kg/h), in order to maintain the growth and secretory activity of their testes. A number of Hx animals were infused with the vehicle.

Acute Experiment

On the 10th day after hypophysectomy, a group of hCG-infused rats received a bolus intraperitoneal injection of 100 IU/kg hCG dissolved in 0.2 ml of 0.9% NaCl, 60 min before the sacrifice. Another group of rats was injected with the vehicle. A number ($n=8$) of rats in each of the two groups had intraperitoneally received together with hCG increasing doses (3, 30 or 300 pmol/kg) of CRH (human and rat, synthetic; Sigma, St. Louis, U.S.A.).

Chronic Experiment

Three groups ($n=8$) of Hx/hCG-infused rats had CRH in the infusion vehicle (3, 30 or 300 pmol/kg/h). The animals were decapitated between 10 00 and 11 00, and their trunk blood and testes were collected.

Testosterone was extracted from heparinized plasma and extracts were chromatographed on Sephadex LH-20 columns. Plasma testosterone concentration (PTC) was assayed by radioimmunoassay (RIA) (T-RIA kit; IRE Sorin, Vercelli, Italy. Sensitivity, 10 pg/tube). Intra- and interassay variations: 6% and 9%, respectively.

The testes of rats from the chronic experiment were employed for morphometric evaluations. The left testis was prefixed *in situ* (11), and then cut into two halves, which were processed for light or electron microscopy, as previously described (3). The right testis was weighed, and its volume was calculated by pyknometry (24); then those obtained from Hx/hCG-infused rats and Hx/hCG-infused rats plus 300 pmol/kg/h CRH were employed to isolate Leydig cells (see below). The following morphometric parameters were calculated on light micrographs of 6 μm -thick paraffin and 0.5 μm -thick epon sections: volume and number of Leydig cells and volume of their nuclei (Weibel-Gomez

method). In the two groups of animals cited above, the following stereologic parameters of Leydig cells were measured on electron micrographs of 600 nm-thick epon sections: volumes of mitochondrial, lipid-droplet, dense-body and peroxisome compartments (differential point counting), and surface area per cell of mitochondrial cristae, smooth (SER) and rough (RER) endoplasmic reticulum and Golgi apparatus (crossing method). The morphometric and stereologic techniques, as well as the sampling procedures were those described by Weibel (35), as detailed by Andreis *et al.* (3).

After weighing, right testes were decapsulated, and Leydig cells were isolated according to Van Damme *et al.* (34), as described earlier (3). Isolated cells from each rat were incubated partly with hCG (5 IU/ml) and partly without hCG. The incubation was carried out in a shaking bath at 37°C for 90 min, in an atmosphere of 95% O₂ and 5% CO₂. At the end of the experiment, the incubation tubes were centrifuged at 4°C and testosterone concentration in the supernatants was measured by RIA, as described above. All the samples were analyzed in the same assay, and intra-assay variation was 6%.

The data were averaged per experimental group and SE was calculated. The statistical comparison of the results was performed by ANOVA, followed by the Student's *t*-test or the Multiple Range test of Duncan.

RESULTS

Acute Experiment

Hypophysectomy decreased PTC (−72%), and the infusion with hCG restored this parameter to the control value. Acute hCG administration elicited a 6-fold surge in PTC. CRH bolus injection did not significantly affect basal PTC. However, CRH, when injected at a dose of 30 or 300 pmol/kg, notably blunted (about −60%) the hCG-induced rise of PTC (Fig. 1).

Chronic Experiment

Again the infusion with hCG annulled the hypophysectomy-induced fall in PTC (−75%). CRH infusion at a rate of 30 or 300 pmol/kg/h decreased PTC by −39% and −75%, respectively (Table 1).

Hypophysectomy caused significant reductions in the volume of testis (−33%), number of Leydig cells per testis (−35%), volume of Leydig cells (−39%) and volume of Leydig-cell nucleus (−39%). The infusion with hCG completely re-

Fig. 1 Effect of bolus intraperitoneal injection of CRH on basal and hCG (100 IU/kg)-stimulated PTC of Hx/hCG-infused rats. Values are means $\pm$ SE ($n=8$). A, $P<0.01$ from control rats; B, $P<0.01$ from Hx rats; C, $P<0.01$ from Hx/hCG-infused rats (Multiple Range test of Duncan)

stored these morphometric parameters to the control values (Table 1). CRH infusion at a rate of 3 pmol/kg/h did not change any of these morphometric parameters in Hx/hCG-treated rats. However, CRH, when infused at a rate of 30 or 300 pmol/kg/h, evoked significant decreases in the volume of testis (-20% and -36%), number of Leydig cells per testis (-25% and -42%), volume of Leydig cells (-28% and -41%) and volume of Leydig-cell nucleus (-34% and -50%) (Table 1).

Stereology (Table 2) showed that the fall in the average volume of Leydig cells of Hx/hCG-treated

rats induced by CRH (300 pmol/kg/h) was coupled with significant decreases in the volumes of the mitochondrial (-38%) and peroxisome compartments (-53%), and in the surface areas per cell of mitochondrial cristae (-45%), SER tubules (-49%) and Golgi cisternae (-59%). The volume of the lipid-droplet compartment underwent a 3.6-fold rise. No changes were noted in the other stereological parameters.

The exposure to hCG caused a 10-fold surge in testosterone production by isolated Leydig cells from Hx/hCG-treated rats. CRH infusion (300

Table 1 *Effects of Chronic Infusion of CRH on the Function and Morphology of Hx/hCG-Treated Rats*

	Sham-operated control rats	Hx rats	Hx/hCG-infused rats	Hx/hCG-infused rats plus CRH (pmol/kg/h)		
				3	30	300
Plasma testosterone concentration (nM)	11.6 ± 1.8	2.9 ± 0.5 ^A	12.8 ± 2.2 ^B	12.3 ± 2.5 ^B	7.8 ± 1.0 ^{B,C}	3.2 ± 0.8 ^{A,C}
Volume of testis (cm ³)	1.75 ± 0.12	1.18 ± 0.07 ^A	1.79 ± 0.15 ^B	1.73 ± 0.14 ^B	1.44 ± 0.11 ^{a,B,C}	1.15 ± 0.06 ^{A,C}
Number of Leydig cells per testis (×10 ⁶)	37.058 ± 2.647	24.118 ± 2.352 ^A	39.706 ± 2.941 ^B	38.235 ± 2.647 ^B	29.704 ± 2.176 ^{a,B,C}	22.842 ± 2.059 ^{A,C}
Volume of Leydig cells (μm ³)	1,079.1 ± 83.7	654.9 ± 44.6 ^A	1,209.3 ± 130.2 ^B	1,142.1 ± 128.2 ^B	874.4 ± 68.8 ^{a,B,C}	716.3 ± 59.5 ^{A,C}
Volume of Leydig-cell nucleus (μm ³)	120.9 ± 6.9	73.7 ± 4.6 ^A	126.8 ± 7.0 ^B	130.1 ± 6.5 ^B	84.1 ± 5.1 ^{a,B,C}	62.8 ± 4.4 ^{A,C}

Values are means ± SE (n=8). a and A, $P < 0.05$ and $P < 0.01$ from control rats; B, $P < 0.01$ from Hx rats; C, $P < 0.01$ from Hx/hCG-treated rats (Multiple Range Test of Duncan)

Table 2 *Effect of CRH Infusion (300 pmol/kg/h) on the Stereologic Parameters of Leydig Cells*

	Hx/hCG-infused rats		Hx/hCG-infused rats plus CRH	
Volume of mitochondrial compartment (μm ³ /cell)	135.1 ±	12.9	84.1 ±	8.2*
Surface area of mitochondrial cristae (μm ² /cell)	60,274.2 ±	7,360.5	33,128.2 ±	4,628.7*
Surface area of SER (μm ² /cell)	67,548.3 ±	7,860.5	35,481.9 ±	5,104.1*
Surface area of Golgi apparatus (μm ² /cell)	6,048.9 ±	675.5	2,447.2 ±	327.8*
Surface area of RER (μm ² /cell)	3,145.8 ±	353.0	2,905.5 ±	359.8*
Volume of lipid-droplet compartment (μm ³ /cell)	2.4 ±	0.5	8.6 ±	1.5*
Volume of dense-body compartment (μm ³ /cell)	4.7 ±	1.1	5.0 ±	1.0*
Volume of peroxisome compartment (μm ³ /cell)	21.3 ±	2.8	9.9 ±	1.7*

Values are means ± SE (n=8). * $P < 0.01$ (Student's *t*-test)

Table 3 *Effect of CRH Infusion (300 pmol/kg/h) on Testosterone Production by Isolated Leydig Cells*

(pmol/10 ⁵ cells/h)	Hx/hCG-infused rats	Hx/hCG-infused rats plus CRH
Basal secretion	50.6 ± 7.2	31.1 ± 5.3*
hCG (5 IU/ml)-stimulated secretion	498.2 ± 69.5	289.5 ± 30.2*

Explanations as in Table 2

pmol/kg/h) significantly reduced both basal (−39%) and hCG-stimulated (−42%) testosterone secretion (Table 3).

DISCUSSION

Our present *in vivo* data indicate that CRH is able to acutely depress the hCG-stimulated (but not the basal) testosterone secretion by Leydig cells (as indicated by PTC) of Hx/hCG-replaced rats. This finding appears to be in keeping with a previous *in vitro* study, showing that CRH does not interfere with hCG binding to rat Leydig cells, but probably inhibits adenyl cyclase/hCG-receptor coupling (33). However, it must be noted that the highest dose of CRH causes an evident decrease in the basal PTC (−26%), which is not significant due to the rather elevated intra-group variability. This point requires further investigation, since an *in vivo* acute effect of CRH on basal testosterone secretion could be conceivably due to the continuous exposure of Leydig cells to circulating gonadotropins.

Chronic CRH infusion causes a marked reduction in the basal PTC of Hx/hCG-administered rats, as well as an evident testis atrophy, coupled with a notable decrease in both the number and average volume of Leydig cells. Leydig-cell atrophy is mainly due to the decrease in the mitochondrial compartment (associated with a parallel fall in the surface area of cristae) and SER. Mitochondria and SER are the two organelles in which the enzymes of testosterone synthesis are located (see 27, for review), and several lines of evidence indicate that in other steroidogenic cells (e.g. adrenocortical cells) the changes in the surface area per cell of mitochondrial cristae and SER membranes are associated with corresponding changes in the activity per cell of some key steroidogenic enzymes (see 25, for reference). Hence, the decrease in these two stereologic parameters may be easily correlated with the reduction in both basal and hCG-stimulated testosterone secretion observed in isolated Leydig cells of rats chronically treated with CRH. Current evidence suggests that gonadotropins, via cyclic-AMP, exert their trophic effect on Leydig cells by enhancing transcription (and translation) of some enzymes of testosterone synthesis (e.g. cytochromes P-450_{sc} and P-450_{17α}) (see 2, 14, 23, 29, for reviews). Since CRH has been reported to inhibit hCG-stimulated cyclic-AMP production (33), it appears reasonable to propose that the mechanism underlying the long-term inhibitory effect of CRH on rat Leydig cells may involve the repression of *de*

novo synthesis of structural and enzymatic proteins. A reduced steroidogenic machinery may explain why after prolonged exposure to CRH, not only hCG-stimulated, but also basal secretory activity is notably decreased in Leydig cells. The striking nuclear atrophy is also in keeping with a depressed protein synthesis in Leydig cells of CRH-infused rats (see 25, 26, for reviews).

Lipid droplets in steroidogenic cells contain cholesterol and cholesterol esters (10, 25), which in Leydig cells are mainly derived from endogenous synthesis (18). Thus, the decreased utilization of cholesterol in testosterone synthesis may account for the conspicuous lipid-droplet accumulation in Leydig cells of CRH-infused rats. Also the atrophy of the Golgi apparatus is in keeping with the reduced steroidogenic capacity of Leydig cells. In fact, some *in vitro* and *in vivo* investigations employing monensin, a disruptor of the Golgi apparatus, show that this organelle is integral to steroidogenesis, at least in adrenocortical cells (7–9, 21).

The decrease in the peroxisomes observed in Leydig cells of CRH-infused rats appears to be a rather puzzling finding, inasmuch as these organelles do not contain steroidogenic enzymes (14, 22). Peroxisomes, at least in hepatocytes, are actively engaged in cholesterol synthesis (5, 27, 31, 32), since they contain 3β-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (19, 20), the rate-limiting enzyme of this process (17). A large body of data suggests that transcription of HMG-CoA reductase gene is negatively regulated by the level of the intracellular cholesterol pool (see 17, for review). In light of these considerations, it could be tentatively proposed that the CRH-induced fall in the number of peroxisomes may be the morphologic counterpart of the reduced endogenous cholesterol synthesis due to the rise in the intracellular cholesterol content (evidenced by lipid-droplet accumulation), which in turn follows the decreased utilization of this precursor in testosterone synthesis.

In conclusion, our present findings indicate that CRH is able *in vivo* to acutely depress hCG-stimulated secretion of testosterone by rat Leydig cells, and, when chronically administered, to inhibit the growth and steroidogenic capacity of these cells.

Caution, however, must be taken in hypothesizing the physiological relevance of the CRH action on Leydig cells. In fact, it must be noted that clear-cut effects were obtained only by administering very high doses of CRH (10 or 100 pmol/rat). We have not actually assayed the concentration reach-

ed by CRH in the plasma of treated rats; however, by assuming 10–12 ml of total plasma volume per rat (28), we can estimate blood levels of CRH of about 10^{-10} – 10^{-9} M. These values exceed that found in normal rats (about 2×10^{-12} M) (36) by more than 2 or 3 order of magnitude. The same is true for the *in vitro* inhibitory effect of CRH, which was observed at a concentration 10^{-7} M (33). However, evidence is available in favor of an auto-crine/paracrine action of CRH on the function of testis: CRH and CRH-mRNA are present in the testis interstitial tissue (30, 37) and CRH binding sites are demonstrated in rat Leydig cells (12, 33). Thus, it seems conceivable that a local production of CRH may easily determine an intra-testicular concentration of this peptide of the order of those found to be effective in our own and previous investigations.

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