

Testicular Steroidogenesis after Human Chorionic Gonadotropin Desensitization in Rats*

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When a single injection of 500 I.U. of human chorionic gonadotropin (hCG) is given to rats there is an initial acute rise of plasma testosterone and of testicular content for both cyclic AMP and testosterone. This response correlates with an increase in both lyase and 17 α -hydroxylase activities. Thereafter both plasma and testicular testosterone decline and do not increase after a second injection of hCG. During this period of desensitization, isolated Leydig cells were insensitive to the steroidogenic stimulatory effect of both hCG and dibutyl cyclic AMP. The post-cyclic AMP block is not due to an alteration of the cyclic AMP-dependent protein kinase but it is correlated with a decrease in both lyase and 17 α -hydroxylase activities of the Leydig cell's microsomes. This decrease is not caused by the absence of the recently described cytosol activator of this enzyme because its addition did not restore the enzymatic activity. Within 60 to 96 h after hCG injection there was a spontaneous increase of both plasma and testicular testosterone and this parallels the recovery of lyase and 17 α -hydroxylase activities. These results suggest that both enzymatic activities are regulated, directly or indirectly, by hCG, and that this is partly responsible for the hCG-induced steroidogenic refractoriness of Leydig cells.

alone (11) and at the conversion of 17-hydroxylated steroids to androgens (12). The former step seems to be impaired in Leydig cells desensitized by a high dose of hCG since the conversion of labeled cholesterol to pregnenolone (13) and pregnenolone formation from endogenous precursors (14) was markedly reduced. In addition, after desensitization by high (13, 14) and low doses (14) of hCG, the conversion of pregnenolone to androgens by desensitized Leydig cells is also impaired, indicating a second steroidogenic block.

This paper reports a decrease of lyase and 17 α -hydroxylase activities in microsomes isolated from rat Leydig cells during hCG-induced desensitization. The possibility that this decrease of enzymatic activities was caused by a diminution of the response to the recently described cytoplasmic activator (12) was also investigated.

MATERIALS AND METHODS

Ninety-day-old Sprague-Dawley rats received a single injection of 500 I.U. of hCG. At each time indicated, half of the animals (8) received an injection of saline (0.9% NaCl solution), and the other half received a second injection of 500 I.U. of hCG. Both groups were killed 2 h later. One testis from each animal was used to determine testosterone and cyclic AMP content as previously described (7). The other testis of each animal of the same group was pooled and Leydig cells were isolated by collagenase dissociation (15). Some of the isolated cells were used to measure the capacity for testosterone production *in vitro* in the presence or absence of hCG or dibutyl cyclic AMP (10). The remaining cells were homogenized in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM dithioerythritol and 0.25 M sucrose. After centrifugation at 10,000 \times g for 10 min, the pellet was saved and used for determination of hCG binding sites by the method described (3). The supernatant was centrifuged at 105,000 \times g for 90 min. The pellet ("microsomes") was suspended in 10 mM Tris-HCl buffer, 1 mM dithioerythritol, and immersed immediately in liquid nitrogen.

To obtain the active cytosol fraction (12), rats were injected subcutaneously with 100 I.U. of hCG and killed with a guillotine 30 min later. Testes were removed, homogenized in 0.25 M sucrose (3 ml/g fresh weight), and the homogenate centrifuged at 105,000 \times g for 60 min. The supernatant from this centrifugation was tested and contained the cytosol activator.

Microsomal Steroidogenic Activity—The catalytic activity of isolated microsomes from the Leydig cells was determined as described by Chasalow (12) with a few slight modifications. Basically the procedure was to incubate 1 ml of 1.0 μ M [7 α -³H]progesterone, 1.0 μ M 17-hydroxy[4-¹⁴C]progesterone, 0.25 mM NADPH in 0.05 M potassium phosphate buffer with the microsomal extracts (70 μ l) of the isolated Leydig cells described above. All incubations were performed both in the presence and absence of the cytosolic activator (100 μ l) noted above (12). The incubation conditions were 37° and open to air. The reaction was initiated by the addition of the microsomal fraction and quenched by the addition of 2 ml of methanol. Time points at 2, 5, 10, and 20 min were obtained. Steroid carriers (20 mg each) for androstenedione and 17-hydroxyprogesterone were added immediately (before storage). At the time a sample was analyzed, distilled water was added and the steroids extracted, chromatographed, and crystallized to constant specific activity as already described (12).

The complex sequence of events which occurs in male rats following a single injection of hCG¹ has been the subject of investigation in several laboratories (1-10). The initial response is an increase in androgen secretion. However, if a second injection of hCG is given within 12 to 48 h of the first, there is no increase in androgen secretion following the latter injection (10). The phenomenon has been called gonadotropin desensitization and has been related to at least three modifications: decrease in the number of membrane receptors (1-5), modification of the coupling system between the receptor and the adenylate cyclase (7), and blockage of steroidogenesis beyond cyclic AMP formation (8-10).

The precise mechanism by which gonadotropins regulate steroidogenesis has not been clarified, but the major sites of action seem to be at the conversion of cholesterol to pregnen-

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¹ The abbreviations used are: hCG, human chorionic gonadotropin; lyase, C-17, C-20 desmolase; pregnenolone, pregn-4-en-3 β -ol-20-one; MEM, Minimum Essential Medium.

Protein Kinase Activity Assay—The method used was similar to that described to measure the protein kinase activity of isolated adrenal cells (16). In brief, isolated Leydig cells were incubated 1 h at 33°C in MEM medium, pH 7.6, containing 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 0.5 mM 1-methyl-3-isobutylxanthine, and 0.5% bovine serum albumin. After incubation the cells were cooled and then centrifuged at $800 \times g$ for 2 min. The pellets were resuspended in 10 mM potassium phosphate buffer, pH 6.5, containing 10 mM magnesium acetate, 0.5 mM 1-methyl-3-isobutylxanthine, and 0.1 M NaCl, and sonicated twice for 5 s at 0°C. The phosphorylation mixture contained histone type A-II (0.25 mg) [γ - 32 P]ATP (12 nmol) in 0.2 ml of buffer in the presence or absence of cyclic AMP. The phosphorylation incubation (5 min at 32°C) was started within 5 min of sonication by addition of 50 μ l of cell homogenate (equivalent to 150,000 to 200,000 cells). The reaction was stopped as described before (16).

Other Methods—Testosterone concentrations in plasma, testis, and in the incubation medium of Leydig cells were estimated by a specific radioimmunoassay (17). Testicular cyclic AMP was purified as described by Mao *et al.* (18). After acetylation (19) the cyclic AMP content was estimated by radioimmunoassay (20).

RESULTS

In Vivo and In Vitro Steroidogenic Refractoriness of Leydig Cells Following hCG Injection—Following a single injection of 500 I.U. of hCG a peak of plasma testosterone is observed within the first 2 h (Fig. 1, circles). Thereafter levels decreased and remained low for about 48 h. (A second, delayed peak of plasma testosterone is observed between 60 and 100 h.) Testicular testosterone (Fig. 2A, open bars) and cyclic AMP (Fig. 2B, open bars) contents are very similar to the pattern of plasma testosterone. Since plasma hCG levels remain high for at least 48 h (3) the foregoing results suggest that after a single injection of hCG, Leydig cells become

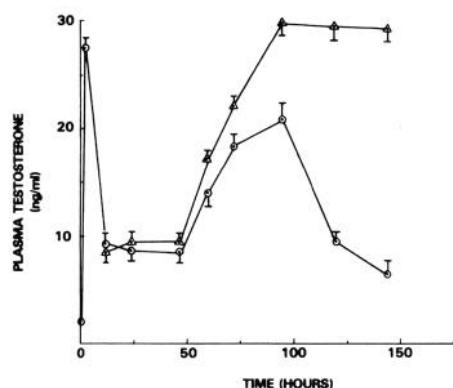


FIG. 1. Plasma testosterone concentrations after hCG treatment. At the start of the experiment each rat was injected with 500 I.U. of hCG. Blood samples were analyzed for testosterone content as shown by the circles. At various times rats were given a second injection and plasma concentration was determined 2 h later (triangles).

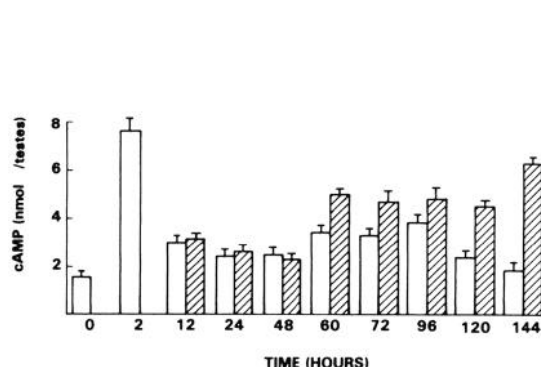


TABLE I
Effect of a single injection of 500 I.U. of hCG on in vitro binding of [125 I]hCG by interstitial cell particles and on protein kinase activity of homogenized Leydig cells

	[125 I]hCG bound	Protein kinase activity (pmol/mg protein/min)		
		Basal	V_{max} (cAMP 10^{-6} M)	K_m of cAMP
	% of control			M
Control	100 \pm 5 ^a	12 \pm 2	42 \pm 4	4.2 $\times 10^{-9}$
Post-hCG 2 h	110 \pm 6	36 \pm 4	46 \pm 6	6.8 $\times 10^{-9}$
Post-hCG 12 h	8 \pm 2	16 \pm 3	42 \pm 2	4.9 $\times 10^{-9}$
Post-hCG 36 h	ND ^b	11 \pm 3	44 \pm 5	4.6 $\times 10^{-9}$
Post-hCG 96 h	12 \pm 3	14 \pm 2	46 \pm 4	3.9 $\times 10^{-9}$

^a Each value shown is the mean \pm S.E. of triplicate determinations of two different experiments (pool of four animals at each time).

^b Not detectable.

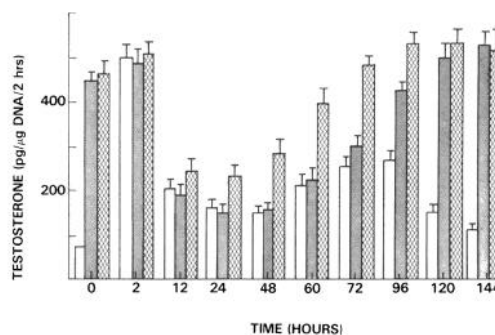


FIG. 3. Effects of a single injection (zero time) of 500 I.U. of hCG on in vitro testosterone production by isolated Leydig cells. Production by cells incubated in the absence of additions are shown by open bars, in the presence of hCG (0.5 μ g/ml) by shaded bars, and in the presence of dibutyryl cyclic AMP (10^{-3} M) by cross-hatched bars.

desensitized to further gonadotropin stimulation. This was confirmed by the fact that between 12 and 48 h after hCG a second administration of the hormone did not produce a significant increase of either plasma testosterone (Fig. 1, triangle) or testicular testosterone and cyclic AMP (Fig. 2, shaded bars).

Under these experimental conditions there are no detectable hCG binding sites on Leydig cells between 12 and 72 h following injection of 500 I.U. of hCG (Ref. 3 and Table I). Therefore, the questions arose whether the concomitant loss of cyclic AMP and testosterone response to further gonadotropin stimulation was only related to the loss of receptors. Fig. 3 shows that desensitized Leydig cells also become resistant to the steroidogenic effect of dibutyryl cyclic AMP. It must be emphasized that *in vitro* steroidogenic responsiveness to dibutyryl cyclic AMP reappears at the same time as the

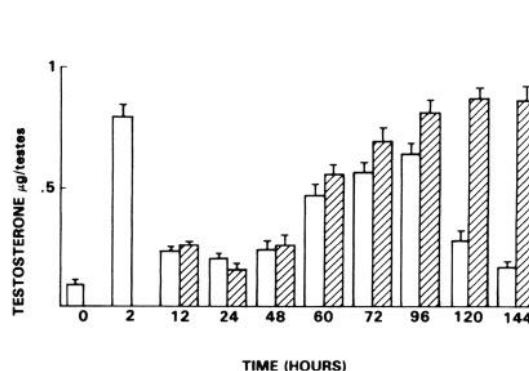


FIG. 2. Testicular contents of cyclic AMP (A) and testosterone (B) at various times after an initial injection of 500 I.U. of hCG. The shaded bars show the amount of cyclic AMP (A) or testosterone (B) present 2 h after a second injection of hCG.

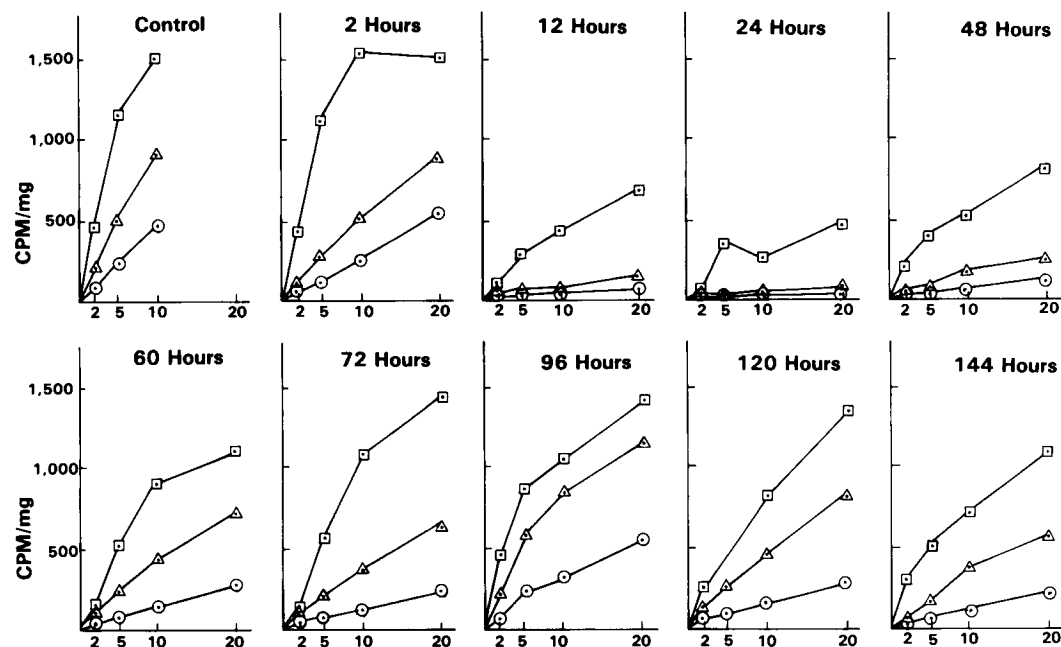


FIG. 4. 17-Hydroxylase and lyase activity in microsomes isolated from Leydig cells from hCG-desensitized rats. Each panel describes the results from a different period after the original treatment. The squares show the 17-hydroxylase activity by the conversion of [3 H]progesterone to 17-hydroxy[3 H]progesterone. The triangles show the lyase complex activity by conversion of [3 H]progesterone to

[3 H]androstenedione. Finally, the circles show the lyase activity by conversion of 17-hydroxy[4 - 14 C]progesterone to [4 - 14 C]androstenedione. In each case the datum shows the final specific activity of the reisolated steroid obtained after the described period of incubation. The details of the method are described under "Materials and Methods."

spontaneous peaks of testosterone in plasma and testis. However full responsiveness to hCG both *in vivo* (Fig. 1) and *in vitro* (Fig. 3) was reached about 48 h later than to dibutyryl cyclic AMP.

Cyclic AMP-dependent Protein Kinase—In order to localize the alteration responsible for the steroidogenic refractoriness to exogenous cyclic AMP, the activity of the cyclic AMP-dependent protein kinase of isolated Leydig cells was investigated (Table I). Basal protein kinase activity was similar in control and in Leydig cells isolated from hCG-treated animals, except in cells isolated 2 h after hCG injection in which the activity was 3-fold higher. However, maximal activation as well as the K_m for cyclic AMP was not very different at the times studied. The absence of a change of cyclic AMP-dependent protein kinase activity during the desensitization period suggested that the alteration in the steroidogenic response to dibutyryl cyclic AMP was located beyond the protein kinase.

Steroidogenic Enzymatic Activity—Within the 1st h after hCG administration, there was an increase of both 17 α -hydroxylase and lyase activities (data not shown) due mainly to the presence of a cytosol activator of these enzymes (12). After 2 h the activity of the lyase was somewhat decreased but that of the 17 α -hydroxylase was not altered (Fig. 4). Thereafter both activities decreased to reach a nadir between 12 and 48 h after the initial treatment. The activity of the 17 α -hydroxylase was decreased about 70% during the desensitization period, while lyase activity was decreased almost 90% with either progesterone or 17 α -hydroxyprogesterone as the substrate. It should be noted that if the 17-hydroxylase activity which is still expressed was required for androstenedione synthesis from progesterone then the relative amount of androstenedione formed from each of the two substrates should have altered and this was not observed. Thus, the 17-hydroxylase activity remaining does not seem to be connected to the lyase complex.

Recovery of both enzymatic activities started 48 h after the initial hCG injection. At 60 h the activity of both enzymes

was about 50% of that of control and at 96 h the recovery was almost complete. It must be noted that recovery of the enzymatic activities parallels the recovery of steroidogenic responsiveness to dibutyryl cyclic AMP.

Effect of Cytosol Activator—Fig. 5 shows some of the data obtained when the cytosol activator was added. Part A shows the effect of the cytosol on microsomes isolated at the same time. Approximately a 50% increase in androstenedione production from progesterone was observed without a corresponding increase in production of 17-hydroxyprogesterone. Part B shows the effect of the cytosol on the microsome sample obtained 2 h after hCG injection. Panel C shows the corresponding data for the 12-h time point. The addition of active cytosol did not restore the activity of either enzyme. However, microsomes obtained 120 h after the initial injection responded to the cytosol with an increase in lyase activity (see Fig. 5D). Therefore, the absence of activity during the 12- to 48-h period cannot be attributed to the absence of the activator nor can the lack of activity be overcome by additional activator. Only after the testosterone response to cyclic AMP or hCG returned, did the response of the microsomes to the cytosol activator also return.

DISCUSSION

Studies from several groups have already shown that hCG-induced Leydig cell desensitization is a complex phenomenon which involves receptor loss (1-6), uncoupling between receptors and adenylate cyclase (7), and blockage of steroidogenesis beyond cyclic AMP formation (8-10). The present observations have shown that the latter alteration was not related to a modification of the cyclic AMP-dependent protein kinase.

Quantitative exploration of the endogenous steroids produced by desensitized Leydig cells (14), as well as the metabolism of labeled pregnenolone (13) has suggested a defect in the lyase complex. The aim of the present study has been to correlate the steroidogenic ability of control and desensitized Leydig cells with the catalytic activity of microsomes in order to determine the nature of the block in the conversion of

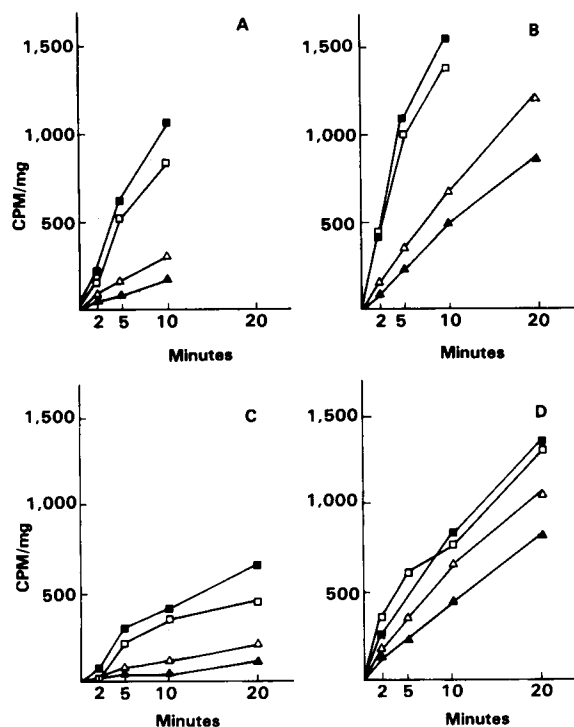


FIG. 5. The effect of the cytosol. These four panels are representative of the data obtained when aliquots of active cytosol were added to the microsomes during the incubation. In each case open marks show the result obtained when cytosol was added. The squares and triangles show the same reaction as in Fig. 4. Panel A shows the effect of the cytosol on freshly isolated microsomes. Panels B, C, and D show the effect of the cytosol on the microsomes obtained 2, 12, and 120 h after hCG desensitization, respectively.

pregnenolone to testosterone induced by hCG treatment.

The procedure used in this paper to measure 17-hydroxylase and lyase activity is very specific. Because the carrier steroids were added at the time of incubation, recovery losses can not occur. The combined chromatography, acetylation, and chromatography procedure would expose the presence of alternate products. Except for small quantities of testosterone in the 20 min of incubation samples from the control, no other steroid products were observed. This was confirmed by the fact that the specific activity of each of the steroid samples was not altered greatly during the crystallization procedure.

The changes in the catalytic activity of the microsomal steroidogenesis enzymes varies in parallel with testosterone production during the desensitization process. Immediately after treatment, the amount of cyclic AMP and testosterone in the testes extracts, the concentration of testosterone in plasma and the ability of the microsomes to synthesize androgens were all elevated. Within 12 h these all decreased and could not be stimulated by a second injection of hCG. A secondary maxima in these parameters occurred between 60 and 96 h after the initial injection. However, during this period a second injection of hCG did not cause an additional increase similar to that initially observed. When 144 h had elapsed, all of these measurements returned to pretreatment levels and a second injection of hCG caused all of the same parameters to increase. The 17-hydroxylase and lyase activities present in the microsomes followed this same pattern. Immediately after injection the activity was increased somewhat. Within 12 h there was a sharp loss in activity which was not restored until 60 h later. However, although the original activity was stimulated by the cytosol activator, it was not until 120 h later that the amount of activity and the response to the activator were restored to the original levels. The decreased synthetic

capability of the isolated microsomes during the early phase was not caused by an absence of the cytosol activator because the addition of the activator did not restore the original activity of the lyase complex. Although a cause and effect relationship has not been demonstrated, the restoration of this response to the activator coincides with the reappearance of hCG binding sites.

The mechanisms responsible for this post-cyclic AMP block are not clear. It has been shown that gonadotropin increases testicular estrogen production (21) and that interstitial cells have specific estrogen receptors (22). Moreover, exogenous estrogens have a direct inhibitory effect on testicular testosterone production (23, 24). Thus, it is possible that increased testicular estrogen production induced by hCG could inhibit testicular steroidogenesis. Indeed, several investigators have noted a decrease in the activity of several enzymes involved in the steroidogenic pathway, namely 17 α -hydroxylase and lyase, after *in vivo* administration of high doses of estrogens for several days (25–27). However, it should be noted that the hCG stimulation of cyclic AMP production by isolated Leydig cells from estradiol-treated animals is significantly higher than that of controls (24). This observation indicated that the alterations induced by estrogens are not related to the pre-cyclic AMP alterations induced by hCG.

In summary, the changes in testicular testosterone production following hCG injection parallel the changes in catalytic activity of the microsomal steroidogenic enzymes. One need not invoke changes in pregnenolone formation by the mitochondria as the only control mechanism for the steroid synthesizing machinery in response to trophic hormone stimulation. Although not yet biochemically defined, a second control sequence exists whereby the microsomal enzymes are regulated.

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