Hormonal Regulation of Gonadotropin Receptors and Steroidogenesis in Cultured Fetal Rat Testes

Abstract. *Gonadotropic activation of the adult rat testis in vitro and in vivo is followed by down-regulation of luteinizing hormone receptors and decreased androgen responses to subsequent hormonal stimulation. In contrast, treatment of cultured fetal testes with gonadotropins and dibutyryl adenosine 3',5'-monophosphate enhanced steroidogenic responsiveness and did not cause the luteinizing hormone–receptor loss and desensitization that is characteristic of the adult gonad. The analysis of gonadotropin receptors and action in cultured fetal testis cells facilitates developmental studies of gonadal function, and has revealed significant differences in the responses of fetal and adult Leydig cells to gonadotropic regulation.*

During sexual differentiation of the mammalian fetus, high concentrations of androgens are necessary for stabilization of the Wolffian duct and masculinization of the external genitalia (*1*). In the adult testis, the ability of Leydig cells to respond to sustained gonadotropic stimulation with increased androgen production is limited by the development of a refractory state associated with loss of luteinizing hormone (LH) receptors and estrogen-dependent decreases in the activity of microsomal cytochrome P-450–dependent enzymes (*2*). The latter changes cause steroidogenic defects in the late androgen biosynthetic pathway, with accumulation of intermediate steroids and impaired testosterone responses to subsequent hormonal stimulation (*3, 4*). Larger doses of gonadotropin cause additional biosynthetic defects prior to pregnenolone production, with an overall decrease in all intermediates of the steroidogenic pathway (*4, 5*). These regulatory properties of the adult Leydig cell have been demonstrated in vivo (*3, 4*) and in vitro (*5*). However, the immature rat testis differs from that of the adult in that LH receptor loss and steroidogenic lesions do not occur after gonadotropic stimulation of the neonatal testis in vivo (*6*).

Since the testis of the neonatal rat still contains the fetal Leydig cells which differentiate during intrauterine life (*7*), these findings suggest that the properties of fetal-neonatal Leydig cells differ from those of the adult cell population which replace them after the third week after birth (*6*). To explore these differences, we analyzed the response of cultured fetal rat testes to gonadotropin stimuli that cause LH receptor loss and steroidogenic lesions in the adult Leydig cell.

All experiments were performed on testes removed from fetal rats at 20.5 days of gestation and placed in multiwell petri dishes. The tissue from four testes was teased with microdissection needles and spread on the surface of the culture dish. Two milliliters of Medium 199 containing 2 m*M* glutamine and 0.1 percent bovine serum albumin were added to each chamber, and the plates were incubated in 5 percent CO_2 and 95 percent air at 37°C for 48 hours. The medium in each well was then replaced with 2 ml of the same medium alone (controls) or the same medium containing, per milliliter, 1, 10, or 100 ng of human chorionic gonadotropin (hCG) (*8*); 1000 ng of ovine LH (oLH) per milliliter (*8*); or 1 m*M* dibutyryl adenosine 3',5'-monophosphate (dibutyryl cyclic AMP). After 24 hours, the solutions were replaced by fresh medium with or without hCG (100 ng/ml) (*9*), and the cells were incubated for a further 3 hours. Media were then analyzed for testosterone, progesterone, 17α-hydroxyprogesterone, and cyclic AMP by radioimmunoassay (*3*), and LH receptors were measured in testis cultures by equilibration with a saturating concentration of ^{125}I-labeled hCG (*4*). Each treatment group consisted of four replicates, and the significance of differences was determined by Student's *t*-test. In addition, collagenase-dispersed Leydig cells from adult rat testes were maintained in primary culture and treated as described above for fetal cultures. As shown in Fig. 1, treatment of fetal testis cultures with hCG or oLH caused prominent, dose-related increases in both basal and hCG-stimulated testosterone production. Incubation with 1 m*M* dibutyryl cyclic AMP also increased both basal and hCG-stimulated testosterone production, though less markedly than the gonadotropins. The basal level of testosterone production after 3 hours of incubation was 32.2 ± 2.0 pg per milliliter per hour (mean ± standard error). After 3 hours of stimulation with hCG, testosterone production increased from the basal value of 122 ± 22 pg/ml-hour to a maximum of 3198 ± 268 pg/ml-hour in cells treated with 1000 ng of oLH per milliliter.

Basal progesterone production was almost undetectable in controls after 3 hours but increased to 6.3 ± 0.9 pg/ml-hour after stimulation with 100 ng of hCG per milliliter. Prior treatment of the testes with 1 ng of hCG for 24 hours caused the highest production rates of progesterone in either basal or hCG-stimulated samples (2.0 ± 1.0 and 8.0 ± 1.5 pg/ml-hour, respectively), but decreased the production of progesterone from testes that received all the other treatments. Basal levels of 17α-hydroxyprogesterone were increased in the testes treated with the hormones or dibutyryl cyclic AMP, and were further stimulated when the testes were incubated for 3 hours with hCG. However, these changes in steroid intermediates were very small compared to the large increments in testosterone production in hCG-stimulated cells, and to the changes seen in gonadotropin-desensitized adult rat testes.

Figure 1 also shows the percentage of free LH receptors (numbers in parentheses) in comparison to controls. No significant differences from controls (0.96 ± 0.14 fmole of bound hCG per testis) were observed except with 1 m*M* dibutyryl cyclic AMP, which increased LH receptors by 47 percent, to 1.41 ± 0.07 fmole per testis ($P < .05$). Stimulation for 3 hours with 100 ng of hCG increased cyclic AMP production in every group, but the cyclic AMP responses in cultures previously treated with hCG or oLH were not significantly different from those of controls. Since testosterone production was highest in testes treated with oLH, the possibility arose that the minor contamination of oLH preparations with follicle-stimulating hormone (FSH) (*8*) could account for the increases above the hCG-treated samples. However, when testis cells were cultured for 24 hours with ovine FSH (10 or 100 ng/ml) with or without 10 ng/ml hCG and then stimulated with hCG (100 ng/ml) for 3 hours, the testosterone responses were similar in both groups.

Studies of adult Leydig cells under similar conditions showed that treatment with 10 ng of hCG for 24 hours significantly reduced hormone-stimulated testosterone production, and 100 ng of hCG completely abolished the ability of the adult cells to be further stimulated by hCG (basal levels, 10.8 ± 1.6 ng; hCG-stimulated, 12.9 ± 1.6 ng/10^6 cells). Treatment with 1000 ng of oLH slightly increased basal testosterone production, but stimulation with hCG did not cause any change; however, treatment with 1 m*M* dibutyryl cyclic AMP caused a de-

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crease in hCG-stimulated testosterone with no change in basal testosterone production. Adult cells treated with 1 and 10 ng of hCG per milliliter showed increases of 100 and 125 percent in progesterone and 17α-hydroxyprogesterone, respectively. The free LH receptors of adult Leydig cells were reduced by about 50 percent after incubation with 10 ng of hCG and were almost completely abolished by 100 ng of hCG per milliliter. These effects of gonadotropins on cultured adult Leydig cells are consistent with those recently observed in vivo and in vitro (*3–5*), and emphasize the prominent negative changes in receptor content and steroid biosynthesis that follow hormonal activation of the adult Leydig cell.

These observations have demonstrated that concentrations of hCG or oLH that down-regulate LH receptors and cause steroidogenic defects in the adult testis (*3–5*) can significantly increase the androgen biosynthetic capacity of fetal Leydig cells. Since testosterone production is elevated in a dose-dependent manner with a reduction in progesterone accumulation, there is no apparent defect in 17α-hydroxylase activity. The minor increase in 17α-hydroxyprogesterone may reflect substrate production beyond the enzymatic capacity of 17-20 desmolase (E.C. 4.1.2. 30). The stimulatory effect of 1 m*M* dibutyryl cyclic AMP on subsequent testosterone responses was relatively small by comparison with the actions of hCG and LH, though this nucleotide caused a significant increase in LH receptors. This could reflect a difference in the sensitivity of the biosynthetic mechanisms for receptors and steroidogenic enzymes to cyclic AMP, although factors such as degradation of the nucleotide during incubation, or possible inhibitory effects of butyrate ion on enzyme induction (*10*), cannot be excluded. The effects of LH and hCG treatment on LH receptor up-regulation were less evident in the cultured fetal cells than in the neonatal testis, in which treatment with gonadotropins in vivo causes a significant increase of 40 to 60 percent in LH receptors after 48 hours (*6*). The less marked effect in the fetal Leydig cell may reflect the shorter duration of exposure to gonadotropin in vitro (24 hours), but remains in marked contrast to the prominent loss of free LH receptors in adult Leydig cells exposed to oLH and hCG in vivo and in vitro (*6*). Since hCG has a similar binding affinity for fetal and adult testes (*11*), some degree of occupancy of the Leydig cell receptors might be expected in fetal testes cultured for 24 hours with hCG at concentrations up to 100 ng/ml. It is therefore possible that positive receptor regulation in the fetal testis was partly obscured by occupancy and that more substantial rises in total receptor number, as induced by treatment with dibutyryl cyclic AMP, also occurred in the hCG-treated testes.

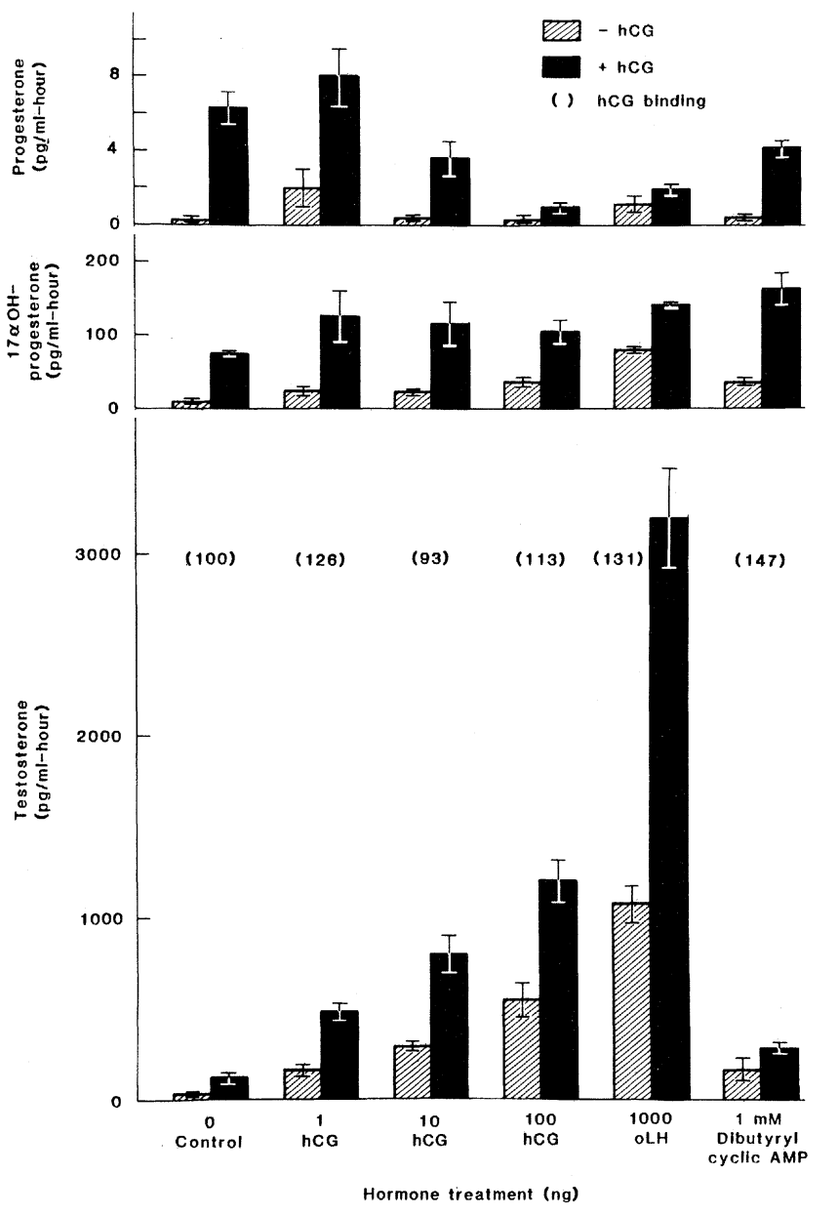

Fig. 1. The effect of treating cultured fetal testes for 24 hours with 1, 10, or 100 ng of hCG per milliliter, 1000 ng of oLH per milliliter, or 1 m*M* dibutyryl cyclic AMP, on testosterone, 17α-hydroxyprogesterone, and progesterone production in response to gonadotropic stimulation. After the 24-hour treatment period, cultures were washed with Medium 199 and steroid hormone production was measured after 3 hours of incubation with medium alone (controls, solid bars) or with medium plus hCG (100 ng/ml) (hatched bars). Concentrations of free LH receptors (shown in parentheses) were measured by a [^{125}I]hCG binding assay. All treatment regimens caused a marked increase in testosterone production after 3 hours of hCG stimulation, to maximum values of 3198 ± 268 pg/ml after incubation with 1000 ng of oLH per milliliter.

The observations described here and previously (*6*) for the adult Leydig cell in vitro are closely similar to the findings of LH receptor down-regulation and steroidogenic defects in the gonadotropin-stimulated adult testis in vivo. Our present finding on the behavior of fetal testes in vitro provides direct evidence that the recently described resistance of the neonatal testis to gonadotropic desensitization is indeed a feature of the fetal Leydig cell. Thus the cultured fetal testis provides a convenient and valid system for comparison with the functional responses of Leydig cells at later stages of gonadal development.

Fetal rat testes in culture were previously used by Picon and Gangerau (*12*)

for studies of the acquisition of sensitivity to LH during development. These workers observed the concomitant appearance of LH-stimulated cyclic AMP and testosterone production on day 15 of gestation, and noted that exposure of 14-day testes to LH did not decrease their subsequent responsiveness to gonadotropic stimulation when differentiation had taken place. In contrast, Paz *et al.* (*13*) observed that the testosterone response of 19-day rat testes decreased during the second and third days of culture with 2 IU of hCG, suggesting the presence of a desensitization process as observed in the adult testis. However, the results of the present study clearly demonstrate that retention of LH receptors and enhanced steroidogenic responses occur in the gonadotropin-stimulated fetal Leydig cell, similar to the responses of the neonatal testis in vivo (*6*). The absence of steroidogenic defects in the fetal testis could be due to the relative lack of estrogen receptors in the immature Leydig cell (*14*), since this lesion appears to be caused through nuclear actions of testicular estrogen following hormonal stimulation (*3–5*) and may only become apparent during postnatal development. Such a feature of the developing gonad could facilitate maintenance of high androgen levels during stimulation by CG, which is detectable in the placenta and maternal circulation of the rat from day 7 of gestation (*15*), prior to sexual differentiation. If the placental hormone is constantly available to the fetal testis, in contrast to the pulsatile adult mode of LH secretion, the ability of the fetal Leydig cell to respond to sustained concentrations of gonadotropin without being desensitized would contribute to the maintenance of elevated androgen production during early development.

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Inhibition of Adrenocorticotropic Hormone Secretion in the Rat by Immunoneutralization of Corticotropin-Releasing Factor

Abstract. *Intravenous administration of rabbit antiserum to ovine corticotropin-releasing factor (CRF) markedly reduced the CRF-induced rise of plasma adrenocorticotropic hormone (ACTH) in intact nonstressed adult male rats while blocking more than 75 percent of the ACTH release observed in rats exposed to ether stress. Furthermore, antiserum to CRF significantly lowered ACTH levels in adrenalectomized animals. These results suggest that endogenous CRF plays a physiological role in regulating ACTH secretion.*

The key role played by the hypothalamus in the regulation of adrenocorticotropic hormone (ACTH) secretion is well established [reviews in (*1*)]. We reported the isolation, characterization, and synthesis of a 41 amino acid peptide from ovine hypothalamic extracts with high potency and intrinsic activity for stimulating the secretion of corticotropin and β-endorphin-like immunoreactivity both in vitro and in vivo (*2–4*). Corticotropin-releasing factor (CRF)–like immunoactivity has been observed in the hypothalamus of the rat, dog, monkey, and human being either by radioimmunoassay or by immunocytochemistry (*5*); it has also been observed in pituitary stalk blood of the rat (*6*). We therefore used antiserum to CRF to investigate the role played by endogenous peptides immunologically related to this CRF in regulating ACTH secretion under various circumstances (*7*).

Adult Sprague-Dawley male rats (230 to 250 g) were given free access to Purina Chow and water. When appropriate, adrenalectomy was performed (by the lumbar approach) in ether-anesthesized animals 3 weeks before the experiment. All animals were equipped with jugular indwelling venous cannulas 36 hours before the experiment and were placed in individual cages in a quiet environment. The cannulas were used both for administration of the treatments and for serial blood sampling.

All three experiments were carried out in the afternoon. In experiment 1A, groups of six intact rats first received 1 ml of normal rabbit serum (NRS) or 1 ml of antiserum to CRF and then, 15 minutes later, saline, synthetic ovine CRF (*8*), or a 3-minute exposure to ether. In experiment 1B, intact rats first received Na_2SO_4-precipitated NRS or antiserum to CRF and then, 1 minute later, saline, CRF, or a 3-minute exposure to ether. In experiment 2, 1.5 ml of NRS or of antiserum to CRF was administered to nonstressed adrenalectomized animals. Since only 0.5 ml of blood was removed during each of the four subsequent samplings, this volume was not replaced.

Figure 1A illustrates the effect of prior treatment with NRS or antiserum to CRF on ACTH secretion (*9*), which was measured 10 minutes after the animal was given saline or 0.45 nmole of CRF or was subjected to ether stress (experiment 1A). In contrast to treatment with NRS, the administration of antiserum to CRF prevented most of the CRF-induced rise in ACTH levels induced by administration of CRF [mean ± standard error (S.E.) of 1305 ± 169 pg/ml versus 182 ± 51 pg/ml; $P \leq .01$] or by ether stress (1729 ± 184 pg/ml versus 363 ± 102 pg/ml; $P \leq .01$). In experiment 1B (Fig. 1B), Na_2SO_4-precipitated NRS or antiserum to CRF was administered in amounts (on a protein basis) equivalent to 1.5 ml of serum, and plas-

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