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GONADOTROPIN STIMULATION OF TESTOSTERONE PRODUCTION IN PRIMARY CULTURE OF ADULT RAT TESTIS CELLS

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Summary: Testis cells from adult hypophysectomized rats were cultured in serum-free medium. Treatment with human chorionic gonadotropin caused an initial increase and a subsequent decline in testosterone production, followed by a recovery in steroidogenesis on day 10 of culture. The recovery in testosterone production was inhibited by the addition of serum in culture media. Luteinizing hormone, dibutyryl adenosine-3',5'-monophosphate or cholera toxin, but not follicle stimulating hormone or prolactin, stimulated testosterone production which was potentiated by a phosphodiesterase inhibitor. This is the first report of a primary culture of adult testis cells with retention of androgen synthetic capacity.

Testicular Leydig cells offer an excellent model for studying the mechanism of action of peptide hormones. These cells have been shown to contain plasma membrane receptor for LH* (1) and respond to LH or hCG* treatment with increases in cAMP* and androgen production during short term incubations (2,3). Although it has been possible to establish murine tumor Leydig cell lines *in vitro* (4-6) and to maintain testis explants from fetal rat and guinea pig (7,8), attempts to establish a primary culture of steroidogenically active Leydig cells were not successful (9). Our preliminary reports indicated that it is possible to establish, from immature rats, a primary culture of testis cells with retention of steroidogenic capacity (10,11). Since these immature testis cells secreted mainly reduced androgens (androsterone and 5α-androstane-3α,17β-diol) and a negligible amount of the bioactive testosterone, we have now modified the culture method to study the effects of gonadotropins, dibutyryl cAMP and cholera toxin on testosterone production by a primary culture of adult testis cells.

Luteinizing hormone, LH; human chorionic gonadotropin, hCG; adenosine-3',5'-monophosphate, cAMP; follicle stimulating hormone, FSH; 1-methyl-3-isobutyl xanthine, MIX.

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Materials and Methods

Adult male rats (Sprague-Dawley, 50-60 days old) were hypophysectomized by Johnson Lab (Bridgeview, IL). Two to three weeks later, the testes were removed, decapsulated, and dissociated in an enzyme solution containing 0.4% collagenase (Worthington Chemical Co., Freehold, NY, 144 U/mg), 10 μg/ml DNAase (2100 U/mg; Grand Island Biological Co., Santa Clara, CA)) and 0.1% bovine serum albumin as described for anterior pituitary cells (12). Briefly, the testicular tissues were incubated at 37°C for 1.5 h and dissociated by repeated pipetting every 30 min with a graded series of micropipettes. At the end of the incubation period, testis cells were washed three times and resuspended. Cell viability was consistently > 95% as measured by Trypan blue exclusion.

Highly purified ovine LH (G3-222B; contains 2.75 NIH-LH-S1 U/mg and 0.001 NIH-FSH-S1 U/mg) and ovine FSH* (G4-211BP, contains 60 NIH-FSH-S1 U/mg and < 0.01 NIH-LH-S1 U/mg) were kindly provided by Dr. H. Papkoff (San Francisco, CA). hCG (CR-121; 13,450 U/mg) was provided by the Center for Population Research. Ovine prolactin (NIH-P-S-13) was obtained from the Pituitary Hormone Distribution Program, NIAMDD. Dibutyryl cAMP, cholera toxin and MIX* were obtained from Sigma Chemical Co. (St. Louis, MO).

Testis cells (0.5 - 1.0 x 10^6 cells/culture) were cultured in McCoy's 5a medium (without serum; Grand Island Biological Co., Santa Clara, CA) supplemented with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and ℓ-glutamine (2 mM). Cell cultures were treated with various hormones and maintained at 37°C under 95% air and 5% CO_2. Media were collected every 2 days for 10 days and replaced with fresh media containing appropriate hormones. Androgen production in the culture media was either measured by direct radioimmunoassay employing antibodies against testosterone-3-oxime-bovine serum albumin (13) or by fractionating the diethyl ether-extracted sample through minicelite columns followed by radioimmunoassay for testosterone, Δ^4-androstenedione, and reduced androgens as previously described (14). Column chromatographic analysis indicated that the present primary culture of testis cells produced mainly testosterone. In a representative sample obtained from hCG-treated cells on day 10 of culture, 4.5 ng/ml of testosterone and 0.31 ng/ml of androstenedione were detected whereas negligible amounts (< 0.03 ng/ml) of dihydrotestosterone, androsterone and 5α-androstane-3α,17β-diol were detected. Since the present testosterone antiserum does not react with Δ^4-androstenedione, comparable results were obtained with or without column chromatography. Data points indicate mean ± S.E. of 4 separate cultures with duplicate radioimmunoassay determinations for each culture. Statistical analyses were done by the Student's t test.

Results

Primary culture of testis cells from adult hypophysectomized rats maintained steroidogenic responsiveness to hCG *in vitro* (Fig. 1) Treatment with increasing concentrations of hCG (0.01 ng-100 ng) caused dose-dependent stimulation of testosterone production on day 2 of culture as compared with control cultures (dashed line). This was followed by a time-related loss of steroidogenic responsiveness to hCG as indicated by the overall decrease in testosterone production on days 4 and 6 of culture. Subsequently, responsiveness recovered and dose-dependent stimulation of testosterone

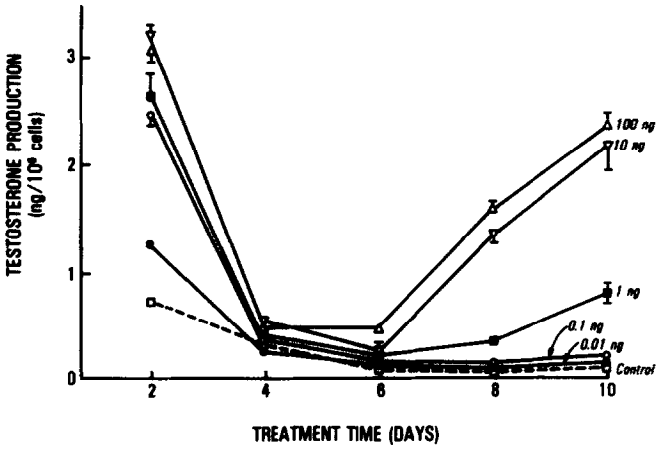


Figure 1 Effect of treatment with increasing concentrations of hCG on testosterone production by primary culture of adult rat testis cells in vitro. The concentrations of hCG (per ml) in cultures are shown. Dashed line indicates control cultures.

production was observed in hCG-treated cells on days 8 and 10 of culture. On day 10 of culture, as low as 1 ng of hCG per ml stimulated a significant increase ($p < 0.01$) in testosterone production whereas 10 ng/ml hCG was maximally effective. The maximal testosterone production on day 10 of culture was comparable to that detected on day 2 of culture, suggesting a near complete recovery of steroidogenic capacity.

To study the inhibitory effects of serum, testis cells were cultured in media containing 0.1 to 10% horse serum. As shown in Fig. 2, serum supplementation did not affect hCG stimulation of testosterone production on days 2 and 4 of culture. The overall steroidogenic responsiveness, however, decreased on day 6 of culture and did not recover up to day 10 of culture. Increasing concentrations of serum caused dose-dependent inhibition of hCG-stimulated testosterone production on days 8 and 10 of culture; 1 or 10% of serum completely inhibited hCG action.

The hormonal specificity of hCG action was also investigated by culturing the testis cells for 8 days without hormone treatment. On day 8 of culture, cells were treated with hCG, LH, FSH or prolactin for 2 days. Both hCG and LH

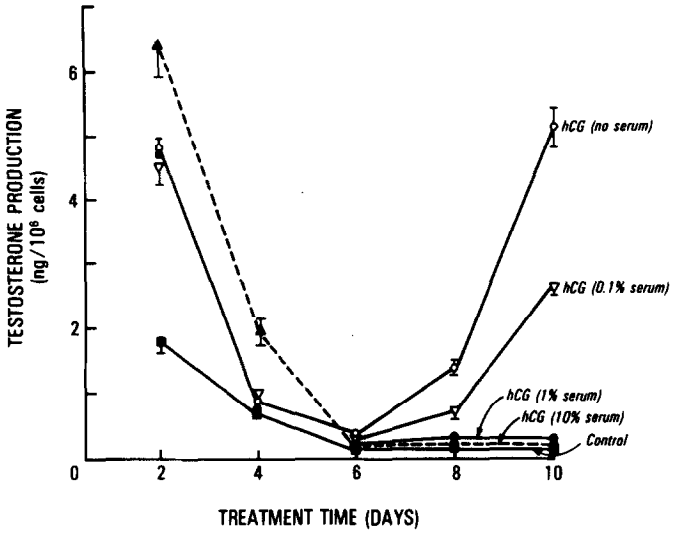
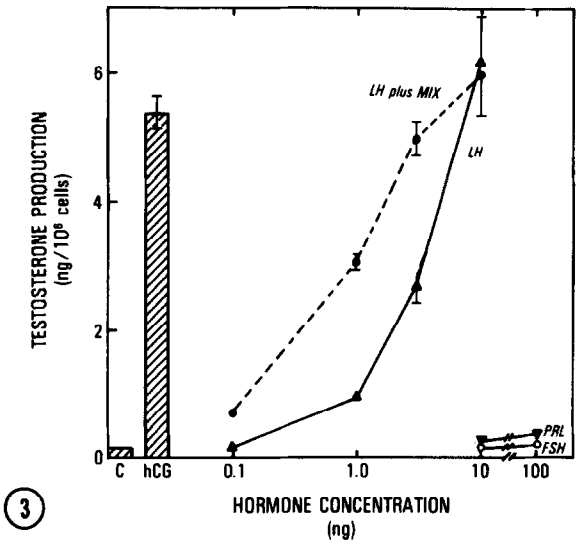


Figure 2 Inhibitory effect of increasing concentrations of serum on hCG (10 ng/ml) stimulation of testosterone production by primary culture of testis cells.

caused substantial increases in androgen production whereas prolactin and FSH were without effect (Fig. 3). The LH effect was shown to be dose-dependent and was further potentiated by concomitant treatment with 0.1mM MIX, an inhibitor of phosphodiesterase activity. The ED_{50} value of LH was decreased from 3.5 to 1 ng/ml, indicating a 3.5 fold increase in Leydig cell responsiveness to LH. Since the potentiating effect of MIX suggested the possible involvement of cAMP, the effect of dibutyryl cAMP and cholera toxin, an agent known to stimulate cAMP production, was also tested (Fig. 4). Both cholera toxin and dibutyryl cAMP stimulated dose-dependent increases in testosterone production and the maximal androgen production was comparable or higher than that induced by hCG.

Discussion

The establishment of a functional primary culture of adult testis cells with retention of androgen biosynthetic potential was reported here. The cultured testis cells respond to treatment with LH and hCG, but not FSH and prolactin, by producing the bioactive androgen testosterone. The hormonal



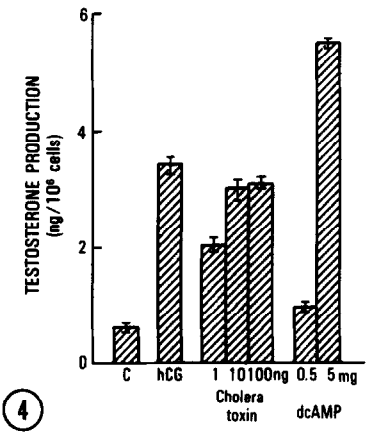


Figure 3 Effect of treatment with hCG, LH, FSH and Prolactin on testosterone production by primary culture of testis cells. Dashed line indicates cultures containing increasing concentrations of LH plus 0.1mM MIX.

Figure 4 Effect of treatment with hCG, cholera toxin and dibutyryl cAMP (dcAMP) on testosterone production by primary culture of testis cells.

specificity of the present culture system conforms with the concept that Leydig cells are the only testis cell type possessing LH receptor. The occupancy of such receptor by LH or hCG results in an increase in androgen biosynthesis. The observed effects of dibutyryl cAMP, cholera toxin and MIX are also consistent with the hypothesis that gonadotropin action on Leydig cells is probably mediated by cAMP.

Earlier failure to maintain androgen synthetic capacity in primary culture of testis cells (9) may be attributed to the use of culture media supplemented with serum. The observed serum inhibition of steroidogenic capacity on days 8 and 10 of culture (Fig. 2) suggests the presence of inhibitory factor(s) in serum which prevents the recovery of steroidogenic responsiveness as found in serum-free cultures. The basis for the observed overall decrease and subsequent "recovery" of steroidogenic responsiveness in serum-free culture is unknown. One possibility is that the Leydig cells adapt

to the in vitro culture environment by synthesizing certain factor(s) essential for de novo androgen biosynthesis. Also, one cannot rule out the possibility that a new population of Leydig cells may acquire steroidogenic capacity in vitro in a time-dependent fashion because recent reports suggest the presence of two populations of Leydig cells in rat testis (15). Both types of Leydig cells contain LH receptors whereas one subtype has substantially higher steroidogenic capacity. Our preliminary results also indicate that testis cells obtained from intact animals do not maintain steroidogenic capacity in vitro. The mechanism underlying this observation is presently unknown. It is possible that the retention of steroidogenic capacity is due to the use of whole testis cells in the present culture which allows interactions between various testis cell types. The possibility of culturing enriched Leydig cells is under investigation.

The demonstration of bioactive testosterone as the major androgen produced in this system is important because only reduced androgens were detected in testis culture of immature rats (10,11). Although one does not know whether the present in vitro system entirely reflects the Leydig cell function in vivo, this culture system provides the first opportunity to study the long term effect of various hormones, trophic agents and drugs on the direct regulation of testis androgen production. Further elucidation of this model system should provide important information regarding the mechanism of action of gonadotrophins on steroidogenesis.

Acknowledgments

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