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Acute stimulation of aromatization in Leydig cells by human chorionic gonadotropin *in vitro*

(estradiol synthesis/testes/aromatase/luteinizing hormone/testosterone metabolism)

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ABSTRACT **Aromatization of testosterone in Leydig cells purified from mature rat testes was assessed. Leydig cells incubated for 4 hr with increasing concentrations of [^{3}H]testosterone exhibited maximal aromatization at 0.6 μM testosterone. At saturating concentrations of testosterone, human chorionic gonadotropin (hCG) acutely stimulated aromatization. This stimulation was first observed at 1 hr, an 8-fold increase being found during a 4-hr incubation. The maximal amount of estradiol produced at saturating concentrations of testosterone and hCG was 1.8 ng per 10^6 cells. These results demonstrate that purified Leydig cells have a high capacity for aromatization and that hCG can acutely stimulate aromatization independently of stimulating testosterone synthesis *in vitro*.**

The capacity of testicular tissue to perform estrogen synthesis has been known for many years. The first demonstration of testicular aromatization of testosterone to estradiol was obtained in studies with homogenates of stallion testes (1). More recently the secretion of estradiol by human, simian, canine, and rat testes has been demonstrated (2, 3). Considerable controversy exists as to the intratesticular site of aromatization and as to which gonadotropin regulates testicular aromatization. *In vitro* studies by de Jong *et al.* (4) suggested that estrogens are synthesized in seminiferous tubules and not in interstitial tissue of adult rat testes. Dorrington and Armstrong (5) reported that Sertoli cells obtained from immature rats can perform estradiol synthesis when maintained in culture in the presence of follicle stimulating hormone (FSH) and testosterone. In contrast, it has been reported that the major site of aromatization in the adult human testis is the interstitial tissue (6). In more recent studies (7, 8) it was found that *in vivo* treatment with luteinizing hormone (LH) or human chorionic gonadotropin (hCG) to adult or immature rats resulted in induction of testicular aromatase activity. Administration of FSH to 15-day-old rats for 3–4 days (8) or to mature rats for 6 days (7) had no effect on testicular aromatase activity. These latter observations suggested that Leydig cells rather than Sertoli cells are involved in testicular aromatization. The present study was undertaken to determine if purified Leydig cells from normal adult rats have the capacity for aromatization and if this step in steroidogenesis can be stimulated by LH/hCG.

MATERIALS AND METHODS

Preparation of Isolated Leydig Cells. Testicular cells were dispersed by treatment of decapsulated testes from 65- to 75-day-old rats (Spartan Research Animals, Haslett, MI) with collagenase as described by Dufau *et al.* (9). Dispersed cells were further purified by metrizamide gradient centrifugation according to a modification of the method described by Conn *et al.* (10). Cells from four testes were resuspended in 2.0 ml of medium 199/0.1% bovine serum albumin, applied to a 40-ml gradient of 0–40% metrizamide (Nyegard, Oslo, Sweden) dissolved in medium 199/0.1% albumin, and centrifuged at 3300 $\times$ *g* for 5 min. One-milliliter fractions were removed from the top of the tube and fractions 25–29 were combined and diluted with 35 ml of medium 199/0.1% albumin; cells were collected by centrifugation for 10 min at 220 $\times$ *g*. The cells were resuspended in medium 199/0.1% albumin. Total cells were counted in a Coulter Counter. The percentage of Leydig cells in fractions 25–29 was >75% as determined by histochemical staining for 3β-hydroxysteroid dehydrogenase (11).

Incubation of Leydig Cells. Aliquots of resuspended cells ($\approx$3 $\times$ 10^5) were incubated in medium 199/0.1% albumin containing indicated amounts of [7-^{3}H]testosterone [25 Ci/mmol (1 Ci = 3.7 $\times$ 10^{10} becquerels); New England Nuclear] with and without hCG (11,000 international units/mg) in a total volume of 1 ml. Control incubations containing everything except cells were run in each experiment. Incubations were carried out for 4 hr or as indicated at 34°C under 95% O_2/5% CO_2. At the end of the incubation period, 2 ml of cold phosphate-buffered saline was added to each vial and the cells plus medium were transferred to glass tubes and put in a boiling water bath for 3 min to denature proteins. The tubes were cooled and centrifuged. The supernatant was used for extraction of steroids and in some experiments the pellet was used for determination of protein by the method of Lowry *et al.* (12).

Extraction and Purification of Steroids. Prior to extraction, [^{14}C]estradiol ($\approx$5000 cpm) and [^{14}C]estrone ($\approx$5000 cpm) were added for determination of recovery. In addition, 50 μg each of nonradioactive testosterone, estradiol, and estrone were added for detection of steroids by iodine vapor on the thin-layer chromatograms. Steroids were first extracted with 9 vol of benzene. Then, phenolic steroids were separated from neutral steroids as described (13). The phenolic extract was acetylated with acetic anhydride/pyridine, 1:2 (vol/vol), for 18 hr at room temperature. The acetylated products were subjected to thin-layer chromatography (ITLC-SA, Gelman) with dichloromethane/ethyl acetate, 99:1 (vol/vol). The radioactive peaks corresponding to estradiol diacetate and estrone acetate were eluted and radioactivity in aliquots was assayed for quantification of the product. Final proof of identity was established by subjecting the remaining eluate to recrystallization to constant specific activity with the appropriate crystalline estrogen acetate. Recovery of ^{14}C-labeled estrogens varied between 60

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Abbreviations: FSH, follicle stimulating hormone (follitropin); hCG, human chorionic gonadotropin; LH, luteinizing hormone (lutropin).

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and 75%. The amount of [^{3}H]estradiol produced, expressed in ng, was calculated from the specific activity of the [^{3}H]testosterone incubated.

In some experiments, equal aliquots of purified Leydig cells were incubated in 1 ml of medium 199/0.1% albumin with increasing concentrations of hCG but no added [^{3}H]testosterone. The amount of testosterone produced by the Leydig cells was determined by radioimmunoassay as described (14).

RESULTS

The effect of increasing concentrations of [^{3}H]testosterone (0.12–1.2 μM) on aromatization (expressed as ng of estradiol) in Leydig cells from adult rats is illustrated in Fig. 1. Maximal aromatization was observed at 0.6 μM testosterone. For subsequent studies on aromatization, Leydig cells were incubated with this concentration of [^{3}H]testosterone. Endogenous testosterone in Leydig cells is released during the purification procedure; in cells obtained after metrizamide gradient density centrifugation, testosterone as measured by radioimmunoassay was found to be negligible. To determine if aromatization could be stimulated *in vitro* by hCG, Leydig cells were incubated for 4 hr with a saturating concentration of [^{3}H]testosterone and increasing concentrations of hCG (0.3–100 pM). hCG stimulated aromatization, with maximal production of 1.8 ng estradiol per 10^6 cells observed at 10 pM hCG (Fig. 2 *upper*). This is approximately an 8-fold increase compared to cells incubated in the absence of hCG. No [^{3}H]estrone was detected in these experiments. When aliquots of the same cell preparation were incubated with increasing concentrations of hCG without added [^{3}H]testosterone, maximal testosterone production also was observed at 10 pM hCG (Fig. 2 *lower*). The maximal amounts of estradiol and testosterone produced by these cells were 0.49 and 21 ng, respectively.

To establish if aromatization in the presence and in the absence of hCG correlated with number of Leydig cells in the incubation medium, increasing numbers of Leydig cells ($1.65–6.6 \times 10^5$) were incubated for 4 hr with saturating concentrations of [^{3}H]testosterone in the absence or in the presence of 30 pM hCG. There was a linear relationship between number of Leydig cells and amount of estradiol produced from testosterone with and without added hCG (Fig. 3). When the time course of hCG stimulation of aromatization by Leydig cells was investigated, a 30-min lag period was seen before a marked increase in aromatization occurred in the presence of hCG (Fig. 4).

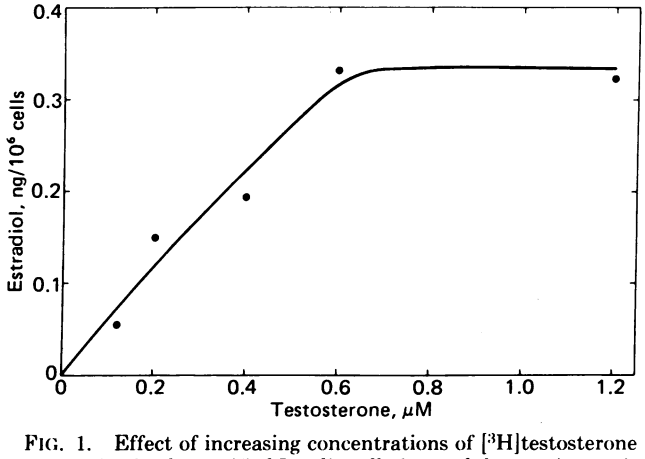

FIG. 1. Effect of increasing concentrations of [^{3}H]testosterone on aromatization by purified Leydig cells from adult rats. Approximately 3×10^5 cells were incubated for 4 hr at 34°C under 95% O_2/5% CO_2 with indicated concentration of [^{3}H]testosterone. Each value represents the mean of duplicate incubations.

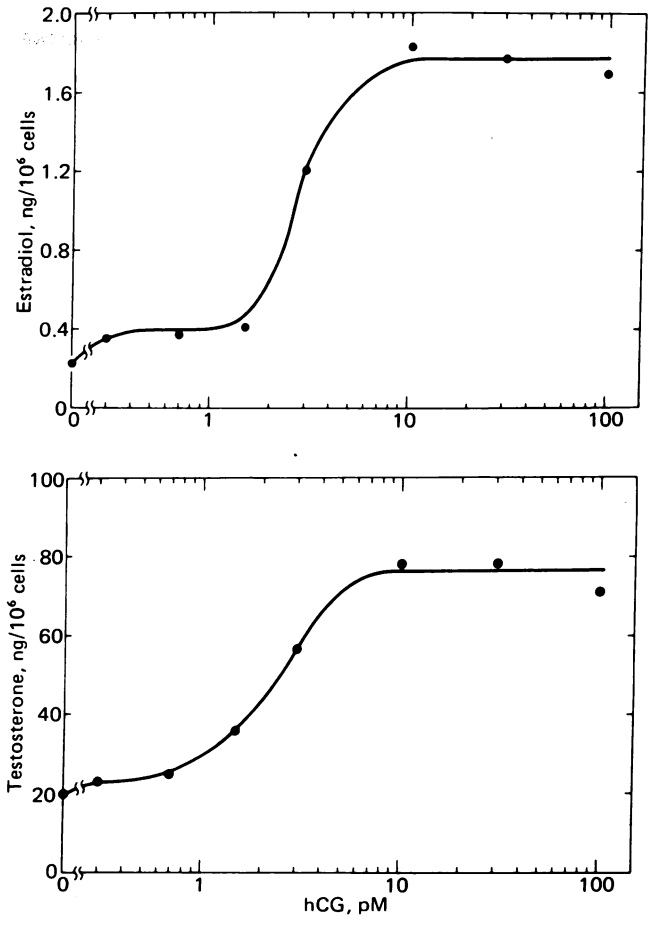

FIG. 2. Effect of increasing concentrations of hCG on *in vitro* aromatization and on testosterone production by purified Leydig cells. (*Upper*) Approximately 3×10^5 cells were incubated for 4 hr at 34°C under 95% O_2/5% CO_2 with 0.6 μM [^{3}H]testosterone and indicated concentrations of hCG. Each value represents the mean of duplicate incubations. (*Lower*) Aliquots of the same cell preparation were incubated for 4 hr at 34°C under 95% O_2/5% CO_2 with increasing concentrations of hCG. Amount of testosterone produced was determined by radioimmunoassay. Each value represents the mean of duplicate incubations.

Because in previous studies (7) we were unable to detect aromatase activity in cell-free testicular homogenates from nontreated mature rats, we investigated if aromatization could be detected in Leydig cells incubated under the same conditions as had been used for testicular homogenates. Leydig cells obtained after metrizamide gradient centrifugation were divided into equal fractions. One fraction was used for incubation with 0.6 μM [^{3}H]testosterone as described above. The other fraction was homogenized and centrifuged at $500 \times g$ for 10 min and aliquots of the supernatant were incubated with 0.6 μM [^{3}H]-testosterone in phosphate-buffered saline containing a NADPH-generating system as described (7). Aromatase activity, expressed as ng of estradiol per mg of protein for a 4-hr incubation, was 0.264 for intact Leydig cells and 0.108 for the cell-free homogenates of Leydig cells. This finding illustrates that incubation of cell-free homogenates with a NADPH-generating system results in markedly decreased aromatase activity compared to incubations of intact viable cells.

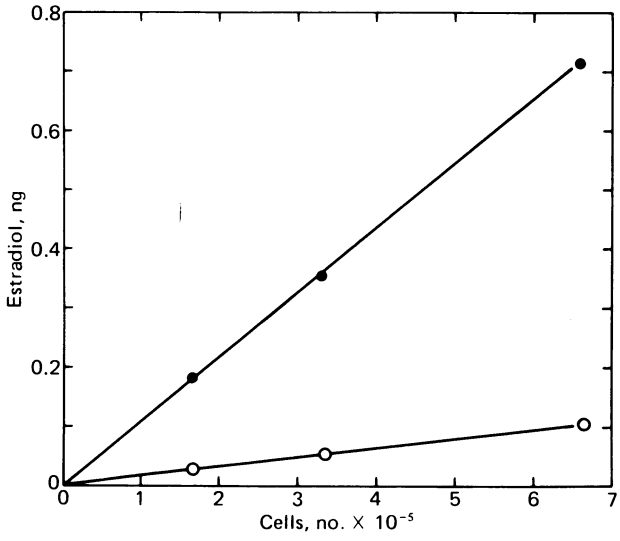

FIG. 3. Effect of increasing concentration of Leydig cells on aromatization. Increasing numbers of Leydig cells were incubated for 4 hr at 34°C under 95% O_2/5% CO_2 with 0.6 μM [^{3}H]testosterone in the absence (○) or in the presence (●) of 30 pM hCG. Each value represents the mean of duplicate incubations.

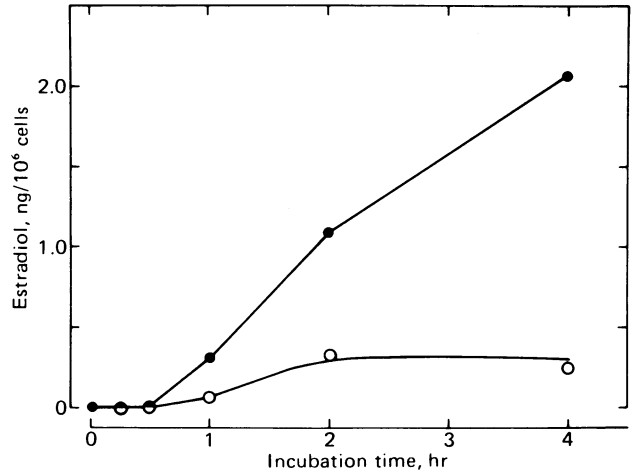

FIG. 4. Relationship of time to *in vitro* hCG stimulation of aromatization by Leydig cells. Approximately 3 × 10^5 cells were incubated for the indicated time at 34°C under 95% O_2/5% CO_2 with 0.6 μM [^{3}H]testosterone in the absence (○) or in the presence (●) of 30 pM hCG. Each value represents the mean of duplicate incubations.

DISCUSSION

The data presented in this report unequivocally demonstrate that Leydig cells from mature rats have the capacity to aromatize testosterone to estradiol. Furthermore, hCG acutely stimulates aromatization in purified Leydig cells. HCG stimulation of estradiol production from testosterone was demonstrated with saturating concentrations of testosterone. This observation indicates that the effect of hCG is on stimulation of the aromatase enzyme(s) and is not due to increased production of testosterone, the substrate for aromatase. Although it should be noted that the concentration of testosterone, 0.6 μM, that resulted in maximal aromatization in the purified Leydig cells is similar to the concentration of testosterone found in rat spermatic vein plasma, 0.4 μM, and in rat interstitial tissue, 0.8 μM, (3, 4), one cannot be absolutely certain that these concentrations of testosterone are saturating for aromatization *in vivo*.

The acute stimulation of aromatization by hCG in Leydig cells was mimicked by dibutyryl cyclic AMP. In a preliminary experiment, the addition of 1 mM dibutyryl cyclic AMP to the medium stimulated aromatization in Leydig cells to the same degree as was observed with 10 pM hCG.

It has been reported (5) that FSH stimulates aromatization in Sertoli cell cultures from immature rats. This finding has led to the proposal that, under the influence of LH, Leydig cells produce testosterone which is then transported to Sertoli cells where in the presence of FSH it is aromatized (15). However, in contrast to the *in vitro* studies on FSH stimulation of aromatization in Sertoli cell cultures, *in vivo* FSH administration to 15-day-old rats for 3–4 days (8) or to mature rats for 6 days (7) did not stimulate testicular aromatase activity. However, daily administration of hCG or LH for 3–6 days to imiature (8) or mature (7) rats markedly stimulated testicular aromatase activity. Furthermore, aromatase activity in the hCG- or LH-treated animals was only demonstrable in isolated interstitial tissue and not in seminiferous tubules. The present study on isolated Leydig cells establishes that there is no need for a two-cell theory to explain aromatization of testosterone in the testis.

The findings in the present study may explain the high ratio of estradiol to testosterone that is found in patients with Klinefelter syndrome in which high LH concentrations are found in serum (16, 17). It is of interest to note that, in Klinefelter syndrome, seminiferous tubules are devoid of Sertoli and germinal cells but interstitial tissue exhibits Leydig cell hyperplasia (18). Recently, Cigorraga *et al.* (19) speculated that a block in testosterone production observed in Leydig cells after treatment with hCG *in vivo* might be due to hCG-stimulated testicular synthesis of estrogens. Our demonstration that Leydig cells stimulated with hCG *in vitro* have a high capacity for estradiol production is consistent with that speculation.

The present study establishes that acute stimulation of testosterone production and acute stimulation of aromatization are independent effects of LH/hCG on Leydig cells *in vitro*. The observed effect of hCG on aromatization in isolated Leydig cells demonstrates that hCG has an acute stimulatory effect at this site in the steroidogenic pathway. Although these results are highly suggestive, the effect of LH/hCG on acute stimulation of aromatization in Leydig cells *in vivo* needs to be determined.

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