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REGULATION OF 5α-REDUCTASE ACTIVITY IN CULTURED IMMATURE LEYDIG CELLS BY HUMAN CHORIONIC GONADOTROPIN

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Summary—The present studies examined the hormonal regulation of 5α-reductase activity in cultured immature rat Leydig cells. Within the testis 5α-reductase was concentrated in the interstitial cell compartment, and among interstitial cells, the enzyme was localized primarily in Band 3 of Percoll density gradients, which contains the majority of Leydig cells. Among various factors reported previously to stimulate testicular 5α-reductase activity when administered *in vivo* to immature rats (LH/hCG, FSH, luteinizing hormone releasing hormone or prolactin), only LH/hCG directly stimulated 5α-reductase activity of cultured immature Band 3 cells. Neither growth hormone which was reported previously to stimulate hepatic 5α-reductase activity, nor insulin, insulin-like growth factor-I, or epidermal growth factor, which have been reported to modulate Leydig cell function, had any effect on 5α-reductase activity of Band 3 cells. These studies suggest that the major factor directly stimulating 5α-reductase activity in Leydig cells during early maturation is LH. However, it is possible that other factors acting indirectly may modulate the maturational rise in 5α-reductase activity.

INTRODUCTION

Maturational changes in testicular 5α-reductase activity play a critical role in regulating the level of testosterone secreted by rat Leydig cells. Between days 20–40 of maturation, Leydig cells actively synthesize testosterone; however, very little androgen is secreted because high 5α-reductase activity converts testosterone to 5α-reduced "metabolites" [17β-hydroxy-5α-androstan-3-one (dihydrotestosterone, DHT), 5α-androstan-3α-17β-diol (3α-diol), 5α-androstan-3β,17β-diol (3β-diol) and 5α-androstan-3α-ol-17-one (androsterone)] [1–4]. Testosterone becomes the major secreted androgen after about 40-days-of-age because of the progressive decline in 5α-reductase activity [2].

With respect to the rat testis, although 5α-reductase has been identified in cells comprising the seminiferous tubules [5, 6], most studies suggest that the enzyme is concentrated in the interstitial compartment [7–9]. Among interstitial cells a recent study suggested that the enzyme is localized in Leydig cells [10].

Factors regulating the early increase and later decline in 5α-reductase activity in rat Leydig cells remain undefined. In immature hypophysectomized rats, LH was reported to stimulate 5α-reductase activity of whole testis homogenates [11, 12] or isolated interstitial cells [13]. A delay in the accumulation of testosterone and a retardation in the maturation of accessory sexual glands following chronic administration of luteinizing hormone releasing hormone (LH-RH) suggested increased 5α-reductase activity by LH-RH [14, 15]. The administration of FSH alone to hypophysectomized immature rats also was reported to stimulate 5α-reductase activity in testicular interstitial tissue [13]. More recently, prolactin was shown to augment LH-stimulated 5α-reductase activity of immature [16] and mature [17] rat testes. In each of the above studies, the hormones were administered *in vivo* to intact or hypophysectomized animals. Thus, whether their effects were mediated through direct or indirect actions on Leydig cells could not be ascertained. To better understand the mode of action of these hormones, their direct effects on 5α-reductase activity in cultured immature Leydig cells were examined. In addition, the effects of other hormones, previously reported to modulate Leydig cell function were examined.

MATERIALS AND METHODS

Animals

Immature Sprague–Dawley rats were obtained from Zivic-Miller Laboratories, Zelienople, Penn.

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Animals were 24–25-days-of-age at the time of sacrifice, or in one instance, 32-days-of-age. Animals were first rendered unconscious in a chamber saturated with CO_2, then killed by decapitation between 0800 and 0900 h. Testes were excised and set at 4°C prior to dispersion with collagenase.

Reagents

Collagenase (Type I), hCG, 8-Br-cAMP, LH-RH, insulin (porcine), dimethyl sulfoxide, penicillin G, streptomycin, NAD^+, NADPH, cycloheximide and spironolactone were from Sigma Chemical Co., St Louis, Mo. Instant thin-layer chromatography sheets (ITLC) were from Gelman Sciences, Ann Arbor, Mich. 4-Methyl-4-aza-3-oxo-5α-pregnan-20(s)-carboxylate (4-MAPC) was kindly provided by Merck, Sharp, and Dohme, Rahway, N.J. Reagent grade organic solvents were from Fisher Scientific Co., Atlanta, Ga. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (F-12), Hepes buffer and $NaHCO_3$ were from Grand Island Biological Co., Grand Island, N.Y. Percoll was from Pharmacia Co., Piscataway, N.J. [1,2,6,7-^{3}H]Testosterone (99 Ci/mmol), [7-^{3}H]testosterone (25 Ci/mmol), [4-^{14}C]dihydrotestosterone (58 mCi/mmol), [7-^{3}H]pregnenolone (23 Ci/mmol) and [4-^{14}C]progesterone (57 mCi/mmol) were from DuPont Co., Boston, Mass. 5α-[1α,2α(n)-^{3}H]Androstane-3α,17β-diol (40 Ci/mmol) and 5α-[1α,2α(n)-^{3}H]-androstane-3β,17β-diol (40 Ci/mmol) were from Amersham Corp., Arlington Heights, Ill. The purity of each labeled steroid was assessed by chromatography or recrystallization. Unlabeled pregnenolone, progesterone, testosterone, dihydrotestosterone, 5α-androstan-3α-17β-diol and 5α-androstan-3β,17β-diol were from Steraloids, Wilton, N.H. and were recrystallized prior to use. FSH (human, LER 8/117) was obtained from Dr Leo Reichert, Albany, N.Y. Insulin-like growth factor-I (IGF-I) was obtained from Amgen, Thousand Oaks, Calif. Epidermal growth factor (EGF) was obtained from Collaborative Research, Bedford, Mass. Prolactin (Prl, ovine, NIH I-5) and GH (rat, NIH I-6) were kindly provided by the National Institutes of Health, Bethesda, Md.

Isolation of interstitial cells and seminiferous tubules

Seminiferous tubules and interstitial tissue were isolated by a wet dissection method [18] as described previously [19]. Assays for 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase (3β-HSD) and 5α-reductase activities were performed on 10,000 *g* supernatants of each compartment as described previously [12].

Isolation of immature Leydig cells

Testes were decapsulated and dispersed with collagenase as described previously [20], but with modifications [10]. Interstitial cells were layered over a 20 ml, continuous 15–60% Percoll gradient [21]; however, instead of using Medium 199 to prepare the gradients, we used DMEM/F-12 (1:1 mixture) containing 1.2 g/l $NaHCO_3$, 15 mM Hepes, 100 U/ml penicillin G and 100 μg/ml streptomycin, and we omitted bovine serum albumin [10]. The gradients were centrifuged at 4°C for 15 min at 3300 *g*. The third band of cells (Band 3, B_3) which contains the majority of Leydig cells and in some earlier experiments, the second band of cells (Band 2, B_2) were isolated and washed thrice using culture medium of DMEM/F-12 (1:1 mixture) containing 1.2 g/l $NaHCO_3$, 15 mM Hepes, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were resuspended in this medium for plating.

The percentage of Leydig cells in B_3 and B_2 was estimated by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) using 5β-androstan-3β-ol-17-one as substrate [22]. Between 30–40% of isolated B_3 cells from animals of this age stained positive for 3β-HSD. This percentage increases to 60–80% after about 30-days-of-age [23].

Culture of Band 3 cells

Approximately 1×10^5 cells/ml were plated into 16 mm 24-well Costar (Cambridge, Mass.) culture dishes, and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 as described previously [10]. 24 h after plating cells were flushed with the original medium and again with fresh medium to remove unattached cells and cellular debris. 1 ml of fresh medium was added to each well and treatment initiated. Media were changed, and cells were retreated every other day.

5α-Reductase and 3β-HSD assays on cultured cells

5α-Reductase activity was estimated by using a procedure similar to that described previously for intact B_3 cells [23], but modified for cultured cells. In brief, following the appropriate treatment period fresh medium was added and again for a second time to remove accumulated steroids. Cells were preincubated in fresh medium in the absence of hormones for 30 min to remove endogenous substrate as described previously for cultured murine Leydig cells [24]. Following this preincubation period, cells were washed two additional times with fresh medium, and the reaction was initiated by the addition of 1 ml of fresh culture medium containing [^{3}H]testosterone (10 μM, 0.5 μCi) and 0.3% dimethyl sulfoxide (final conc.). We estimated the amount of substrate produced during the 30 min preincubation period and determined that testosterone levels were $< 0.01\%$ of the concentration of substrate added, suggesting negligible dilution of our exogenously added substrate. Also, an intact cell assay procedure is justified because the plasma membrane does not limit the entry or association of exogenously added steroid with intracellular membrane-associated enzymes [25].

Table 1. Localization of 3β-HSD and-reductase activities in immature rat testes

Testicular compartment	Enzyme activity: 3β-HSD (nmol progesterone/ min/mg protein)	5α-reductase (nmol DHT + 3α- and 3β-diol/ min/mg protein)
Intact testis	0.38	0.22
Interstitial cells	2.86	1.55
Seminiferous tubules	0.014	0.020
IC/ST	206	78

Animals were 32-days-of-age.

The reaction period for 5α-reductase was for 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The reaction was terminated by the addition of 0.1 ml of 1 N NaOH. 100 μg each of testosterone, [^{14}C]dihydrotestosterone (approx. 4000 cpm), 3α-diol and 3β-diol were added to serve as carriers and for recovery estimates. In separate wells about 4000 cpm each of [^{3}H]5α-androstan-3α,17β-diol and [^{3}H]5α-androstan-3β,17β-diol were added for recovery estimates of these steroids. Samples were extracted with 5 vol of diethyl ether, and the residues of ether extracts were chromatographed by ITLC using a solvent system of chloroform:methanol (99.0:1.0, v/v). The R_f for DHT in this solvent system was 0.70, and the R_f for 3α- and 3β-diol (which co-localized in one area) was 0.31. The products DHT, 3α-diol and 3β-diol were localized using iodine vapors, cut out and counted in Omnifluor-toluene.

To determine 3β-HSD activity in cultured Band 3 cells, following the appropriate culture period, media were changed and cells preincubated for 30 min with fresh media as described for 5α-reductase assay. Following removal of the preincubation media, the reaction was initiated by the addition of fresh medium containing [^{3}H]pregnenolone (10 μM, 0.5 μCi), 10 μM spironolactone (to inhibit 17α-hydroxylation of the substrate pregnenolone and product progesterone [26]) and 0.3% dimethyl sulfoxide (final conc.). The incubation time and conditions and the method used to terminate the reaction were identical to that described for 5α-reductase assay. Following the reaction 100 μg each of pregnenolone and [^{14}C]progesterone (approx. 4000 cpm) were added as carriers and to estimate recoveries. Samples were extracted with 5 vol of diethyl ether and the residues of ether extracts were chromatographed by ITLC in chloroform:methanol (99.5:0.05, v/v) as the solvent system. The R_f for progesterone was 0.77. Progesterone was localized with iodine vapors, and this area was counted on Omnifluor-toluene.

Table 2. Ratio of 3β-HSD histochemical staining, 3β-HSD and 5α-reductase activities in cultured immature Band 3 vs Band 2 cells

Enzyme activity	No. of experiments	Activity ratio (B_3/B_2)
3β-HSD histochemical[a]	24	8.1
3β-HSD biochemical[b]	7	4.9
5α-Reductase[b]	7	6.7

Animals were 25-days-of-age. [a]Cells were stained for 3β-HSD on the day of isolation. [b]Assays were performed on day 3 of culture.

Testosterone determination

Testosterone levels in the medium were estimated by radioimmunoassay as described previously [20].

Statistics

Treatment groups were compared by analysis of variance. If F-values were significant, differences were determined using Duncan's new multiple range test. For comparisons of two treatment groups, Student's t-test for non-paired means was utilized.

RESULTS

Localization of 3β-HSD and 5α-reductase activities in immature rat testis

The high interstitial cell/seminiferous tubule ratio (206) for 3β-HSD activity demonstrates that this enzyme is an effective marker for Leydig cells (Table 1). The interstitial cell/seminiferous tubule ratio for 5α-reductase of 78 suggests concentration of the enzyme within the interstitial compartment; however, the lower ratio when compared to 3β-HSD suggest some 5α-reductase localization in seminiferous tubules.

Localization of 3β-HSD and 5α-reductase activities in Band 3 vs Band 2 cells

The number of positive 3β-HSD staining cells, 3β-HSD and 5α-reductase activities all were higher in B_3 cells (Table 2). The similar activity ratios in B_3 vs B_2 cells for 5α-reductase, 3β-HSD and positive 3β-HSD staining cells suggest that 5α-reductase is concentrated in Leydig cells among cells comprising interstitial tissue.

Table 3. Effect of hCG or 8-Br-cAMP on testosterone accumulation in immature Leydig cells cultured without or with 4-MAPC

Treatment	ng Testosterone/10^5 cells: No 4-MAPC	+1 μM 4-MAPC
None	0.476 ± 0.086	2.218 ± 0.156‡
10 mIU hCG	0.822 ± 0.052*	61.142 ± 2.257†‡
0.1 mM 8-Br-cAMP	1.356 ± 0.077*	61.376 ± 1.587†‡

Each value represents the mean ± SE of 4 determinations.
Band 3 cells from 25-day-old rats were treated 24 h after plating, and media were collected 48 h thereafter for quantitation of testosterone by radioimmunoassay.
*$P < 0.01$ when compared to no 4-MAPC control; †$P < 0.01$ when compared to +4-MAPC control; ‡$P < 0.01$ when compared to corresponding treatment groups cultured without 4-MAPC.

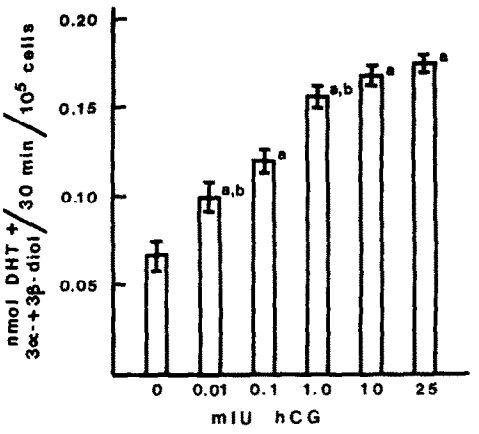

Fig. 1. Effect of increasing hCG concentrations on 5α-reductase activity in cultured immature Leydig cells. Each treatment group represents the mean ± SE of 4 separate determinations. Cells were washed, media changed and treatment initiated 24 h after plating. 5α-Reductase activity was determined 48 h thereafter. $^{a}P < 0.01$ when compared to control; $^{b}P < 0.01$ when compared to immediately lower hCG concentration.

Effect of 5α-reductase inhibitor on testosterone accumulation of cultured immature Leydig cells

In the absence of the 5α-reductase inhibitor, 4-MAPC, very little testosterone accumulated, even following treatment with hCG or 8-Br-cAMP (Table 3). However, in the presence of 4-MAPC much higher levels of testosterone accumulated in the basal state and following treatment with hCG or 8-Br-cAMP.

Effect of hCG or 8-Br-cAMP on 5α-reductase activity in cultured immature Leydig cells

Basal 5α-reductase activity on day 3 of culture was 0.068 ± 0.006 nmol DHT and 3α-+3β-diol/30 min/10^5 cells (Fig. 1). Activity increased in relation to the dose of hCG administered and peaked (approx. 2.5-fold above control) at about 10 mIU

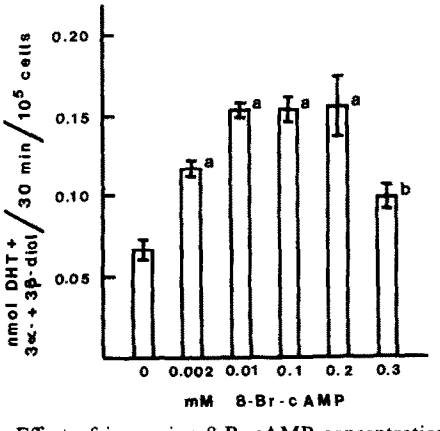

Fig. 2. Effect of increasing 8-Br-cAMP concentrations on 5α-reductase activity in cultured immature Leydig cells. See legend to Fig. 1 for details of treatment. $^{a}P < 0.01$ when compared to control; $^{b}P < 0.05$ when compared to immediately lower 8-Br-cAMP concentration.

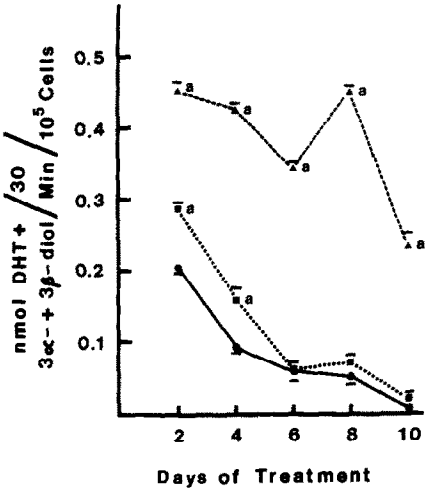

Fig. 3. Effect of time of exposure to hCG or 8-Br-cAMP on 5α-reductase activity in cultured immature Leydig cells. Each value represents the mean ± SE of 4 separate determinations. Cells were washed, media changed and treatment initiated 24 h after plating with media change and retreatment every other day of culture. —●—, control; --▲--, 10 mIU/ml hCG; ··■··, 0.1 mM 8-Br-cAMP. $^{a}P < 0.01$ when compared to control of comparable treatment period.

hCG. To determine whether the increase in 5α-reductase activity by hCG involved the net synthesis of protein, untreated and hCG-treated B_3 cells were treated with 10 μg/ml cycloheximide, and activity was measured 24 h thereafter. However, because cycloheximide inhibited both basal and hCG-treated 5α-reductase activity, we were not able to ascertain whether increase in 5α-reductase involved new protein synthesis (data not shown).

5α-Reductase activity also was responsive to increasing 8-Br-cAMP concentrations (Fig. 2). However, after peaking at 0.01 mM 8-Br-cAMP,

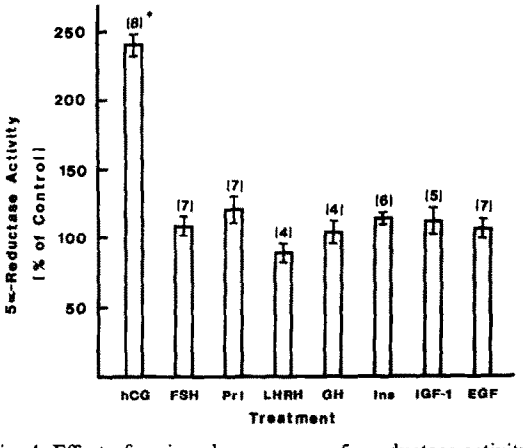

Fig. 4. Effect of various hormones on 5α-reductase activity in cultured immature Leydig cells. See legend to Fig. 1 for details of treatment. Concentration of hCG was 10 mIU/ml; concentration of insulin was 1 μg/ml; concentration of FSH, prolactin, LHRH, GH, IGF-I and EGF was 100 ng/ml. *Represents number of experiments for each hormone examined.

activity declined following addition of 0.3 mM 8-Br-cAMP.

Effect of long-term treatment with hCG or 8-Br-cAMP on 5α-reductase activity in cultured immature Leydig cells

5α-Reductase activity of control B_3 cells progressively declined during the 10-day treatment period (Fig. 3). Activity increased with 0.1 mM 8-Br-cAMP following 2 and 4 days of treatment; however, stimulation was not sustained following 6 days of treatment and thereafter. In the presence of 10 mIU hCG, high 5α-reductase activity was maintained for 8 days of treatment. Activity declined from the 8-day level on day 10 of treatment, but activity was still higher than control.

Effect of hormones alone or with hCG on 5α-reductase activity in cultured immature Leydig cells

We examined whether other hormones (FSH, prolactin, LH-RH, GH, insulin, IGF-I or EGF) each added alone to cultured B_3 cells had any effect on 5α-reductase activity. Among the hormones tested, only hCG stimulated 5α-reductase activity on day 3 of culture (Fig. 4). We examined also whether these same hormones could augment the increase in 5α-reductase activity exhibited by hCG. However, when each hormone was added with hCG to cultured B_3 cells, there was no additional increase in 5α-reductase activity above the level observed with hCG alone (Fig. 5). For each hormone other than insulin (which was added at a concentration of 1 μg/ml) we examined both lower and higher (up to 500 ng/ml) concentrations (added alone or with hCG) for up to six days of treatment, but observed no effect on 5α-reductase activity (data not shown).

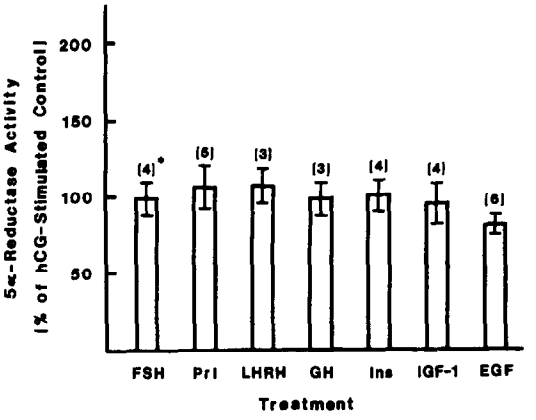

Fig. 5. Effect of various hormones on hCG-stimulated 5α-reductase activity in cultured immature Leydig cells. See legends to Fig. 1 and Fig. 4 for details of treatment and concentrations of hormones added, respectively. *Represents the number of experiments for each hormone examined.

DISCUSSION

The present studies demonstrate the suitability of cultured Leydig cells from immature rats as a model to examine 5α-reductase regulation in rat testes. Among several hormones tested, only hCG significantly stimulated 5α-reductase activity in cultured cells.

The present results support previous studies demonstrating that 5α-reductase activity in rat testes is concentrated in interstitial cells [7–9], and that among interstitial cells, the enzyme is concentrated in Leydig cells [10]. It is interesting that in using 3β-HSD staining to identify Leydig cells, the B_3/B_2 staining ratio was consistently higher than the B_3/B_2 activity ratio for 3β-HSD. This may suggest that the 3β-HSD staining procedure does not identify all Leydig cells in B_2 or that this method underestimates 3β-HSD activity in B_2. The B_3/B_2 activity ratio for 5α-reductase was intermediate between that of 3β-HSD histochemical staining and 3β-HSD activity.

To our knowledge, the present results represent the first *in vitro* studies demonstrating stimulation of 5α-reductase in B_3 cells by LH/hCG, and they support previous *in vivo* studies which showed increased enzyme activity by LH in homogenates of whole testis [11, 12] and interstitial cells [13]. Within the first 2–4 days of stimulation, the effects of hCG were mimicked by 8-Br-cAMP; however, after this period of exposure, 8-Br-cAMP was no longer as effective as hCG. This may suggest that long-term maintenance of the enzyme requires a second messenger other than cAMP. Whether the increase in 5α-reductase activity by hCG involves protein synthesis and/or stabilization of the existing enzyme pool remains to be determined.

Our failure to observe a direct effect of LH-RH on 5α-reductase in cultured immature B_3 cells suggests that the previous reported stimulation in immature rat testes following treatment of rats with LH-RH [14, 15] was mediated indirectly by the release of pituitary LH, even though LH-RH receptors have been localized in rat Leydig cells [27].

Recent studies showed that prolactin administration to immature [16] or mature hypophysectomized rats [17] augmented LH-stimulated 5α-reductase in testes; however, we were unable to demonstrate a direct prolactin effect in cultured immature B_3 cells when administered alone or with hCG. Although prolactin receptors have been localized in Leydig cells of rat testes [28] and prolactin was proposed to have direct effects on Leydig cell 5α-reductase [16], the results of the present study suggest that the action(s) of prolactin is mediated indirectly: (1) through another cell type localized within the interstitium which is removed during density gradient purification, (2) through a cell type comprising the seminiferous tubules or (3) through another organ.

It has been reported that GH stimulates 5α-reductase activity in cultured rat hepatocytes [29]; however, GH had no effect on 5α-reductase in cultured immature B_3 cells in the present studies. Also, IGF-I

which mediates many actions of GH had no effect in the present studies. Similarly, neither EGF or insulin had any effect of 5α-reductase activity in the present studies.

A previous study showed that FSH administration to immature hypophysectomized rats increased 5α-reductase activity in interstitial cells [13]. The lack of a direct effect of FSH on 5α-reductase activity in cultured immature Leydig cells in the present studies suggest that the actions of FSH were mediated indirectly by a factor(s) secreted by Sertoli cells since FSH actions in testes appear to be mediated through Sertoli cells [30]. The presence and nature of such a factor remain to be ascertained.

Among the various factors examined, none inhibited either basal or hCG-stimulated 5α-reductase. Whether such a factor is present to selectively inhibit 5α-reductase activity remains to be determined.

In summary, the present studies demonstrate that cultured immature Leydig cells are a suitable model to examine 5α-reductase regulation in rat testes. These studies suggest that the primary factor responsible for the early maturational increase in 5α-reductase activity of rat testes is LH. The actions of LH appear, in part, to be mediated by cAMP. Whether another factor(s) is involved in the early maturational increase in 5α-reductase activity, and the mechanism(s) responsible for the later selective decline in the testicular enzyme remain to be determined.

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