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EVIDENCE THAT LEYDIG PRECURSORS LOCALIZE IN IMMATURE BAND TWO CELLS ISOLATED ON PERCOLL GRADIENTS

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Summary—The present studies examined responses to hCG and/or insulin of 3β-hydroxy-5-ene-steroid dehydrogenase and steroid $5\rightarrow4$-ene-isomerase activity (3β-HSD) in cultured Band 2 and Band 3 cells from 25- to 40-day-old rats isolated on Percoll gradients. In Band 2 cells, from 25-day-old rats enzyme activity increased about 3- and 2.5-fold, after 6 days of exposure to hCG or insulin, respectively. However, hCG did not stimulate enzyme activity in Band 2 cells from 30-, 35- and 40-day-old animals, and responses to insulin alone or insulin plus hCG declined with age. In Band 3 cells only insulin increased enzyme activity at each age. Neither hCG or insulin altered DNA levels in Band 2 or Band 3 cells, suggesting that increased activity in Band 2 cells from 25-day-old rats was not due to cellular replication. However, hCG increased the number of cells staining positive for 3β-HSD about 4-fold in Band 2 cells from 25-day-old rats. Insulin did not increase the number of positive staining cells in Band 2 and Band 3 cells from 25-day-old rats, suggesting that its major effect was to increase enzyme activity in existing cells. These results suggest that during a limited period of maturation precursor cells in Band 2, which are undetected by histochemical staining for 3β-HSD, can be converted to Leydig cells in culture by hCG.

INTRODUCTION

Rat or mouse Leydig cells localize generally in two or more bands when dispersed interstitial cells are centrifuged on various density gradients [1–3]. The band localizing in the denser (average density about 1.07 on Percoll gradients) region (referred to as Population II or Band 3) contains a higher concentration of Leydig cells which are highly responsive to LH/hCG with respect to testosterone formation [2–4]. The band localizing in the less dense (average density about 1.05 on Percoll gradients) region (referred to as Population I or Band 2) contains a lower Leydig cell concentration which produces less testosterone in response to LH/hCG [2–4]. It has been reported that this difference in testosterone producing capacity between these two populations of cells is due in part to differences in steroidogenic enzyme activities [5, 6] and that Band 2 Leydig cells give rise to Leydig cells in Band 3 during sexual maturation [7]. However, other studies suggest that adult Leydig cells localizing in the less dense region represent damaged cells [8, 9] or that Leydig cells from both regions exhibit similar steroidogenic capacity [10], thereby implying Leydig cell homogeneity. Furthermore, it was reported that adult mouse Leydig cells appearing damaged in Band 2 of Percoll gradients actually were indeterminant connective tissue cells that may be Leydig cell precursors [11].

Because the banding pattern of rat interstitial cells on Percoll gradients and the pattern of hCG-stimulated androgen production by cultured Band 2 and Band 3 cells change during maturation [12, 13], we examined the response to hCG and/or insulin of 3β-HSD activity in cultured Band 2 and Band 3 cells between 25–40 days of age. We provide evidence that precursor cells localize in Band 2, and during a limited period of maturation they can be converted to Leydig cells in culture by hCG.

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Abbreviations: 3β-HSD = 3β-hydroxysteroid dehydrogenase-isomerase; hCG = human chorionic gonadotropin; ITLC = instant thin layer chromatography; DMEM = Dulbecco's modified Eagle's medium; F-12 = Ham's F-12 nutrient mixture; HEPES = N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid).

MATERIALS AND METHODS

Animals and reagents

Sprague–Dawley rats were purchased from Zivic–Miller Lab., Zelienople, Pa. They were rendered unconscious in a CO_2 chamber, then killed by decapitation between 0800 and 0900 h. Testes were excised, decapsulated and dispersed with collagenase to obtain interstitial cells [14]. Collagenase (Type I), hCG, dimethyl sulfoxide, penicillin G, streptomycin, NAD^+, insulin (porcine), nitroblue tetrazolium, spironolactone and 5β-androstan-3β-ol-17-one were from Sigma Chemical Co., St Louis, Mo. Instant thin-layer chromatography (ITLC) sheets were from Gelman Sciences, Ann Arbor, Mich. Dulbecco's modified Eagle's medium (DMEM), Ham's nutrient mixture (F-12), *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethane sulfonic acid] (HEPES) and $NaHCO_3$ were from Grand Island Biological Co., Grand Island, N.Y. Percoll was from Pharmacia Co., Piscataway, N.J. [7-^{3}H]-Pregnenolone (23 Ci/mmol) and [4-^{14}C]progesterone (57 mCi/mmol) were from DuPont Co., Boston, Mass. Pregnenolone and progesterone were from Steraloids, Wilton, N.H.

Isolation and culture of Band 2 and Band 3 cells

Interstitial cells were layered over a 20 ml continuous 15–60% Percoll gradient and centrifuged at 3300 *g* for 15 min to isolate Band 2 and Band 3 cells [13]. Cells were washed thrice in culture medium consisting 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. Using histochemical staining for 3β-HSD with 5β-androstan-3β-ol-17-one as substrate to identify Leydig cells [15], we observed that approx. 40% of Band 3 cells from 25-day-old rats stained positive, and this percentage increased to approx. 60% or greater in Band 3 cells from older animals. The percentage of Band 2 cells staining positive for 3β-HSD for all age groups was between 5 and 10%. Cells were suspended in the same culture medium (conc. of 10^5/ml) and 1 ml of cells was plated into each 16 mm diameter well of a 24-well Costar culture dish [13]. Media were changed 24 h after plating and treatment initiated. Media were changed and cells retreated every other day. Assays were performed after a 6-day treatment period, because preliminary studies showed maximal responses following 4 or 6 days of exposure to 10 mIU/ml hCG or 2 μg/ml insulin.

3β-Hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase (3β-HSD) assay in cultured cells

Following 6 days of treatment, media were changed twice and cells preincubated for 30 min in fresh media to remove accumulated steroids. Media were changed two additional times, and the reaction was initiated by adding 1 ml of fresh medium containing [^{3}H]pregnenolone (10 μM, 0.5 μCi), 10 μM spironolactone (to inhibit 17α-hydroxylation of pregnenolone and progesterone [6] and 38 mM dimethyl sulfoxide as described previously [17]). The reaction period was 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and was terminated by adding 0.1 ml of 1 N NaOH. Pregnenolone (100 μg) and [^{14}C]progesterone (about 4000 cpm and 100 μg) were added as carriers and to estimate recoveries. Samples were extracted with 5 vol of diethyl ether and chromatographed by ITLC in chloroform:methanol (99.5:0.5, v/v). Progesterone was localized by iodine vapors and counted in Omnifluor–toluene. DNA content of each well was determined by the diphenylamine procedure [18], after cells were solubilized with 0.1 N NaOH.

Histochemical staining for 3β-hydroxysteroid dehydrogenase

Approximately 4×10^5 cells/4 ml were plated into 35 mm diameter culture dishes containing 22 mm diameter glass coverslips. Following 6 days of treatment, coverslips were air-dried to permeabilize the plasma membrane and stained for 3β-HSD using 5β-androstan-3β-ol-17-one as substrate [15]. Nonspecific staining was determined by omitting substrate. The results are presented as the total number of positive staining cells per 10 random fields.

Statistical analysis

Treatment groups were compared by analysis of variance. Differences among treatment groups were determined using Duncan's new multiple range test. A P value of < 0.05 was considered statistically significant.

RESULTS

Effect of hCG and/or insulin on 3β-hydroxysteroid dehydrogenase-isomerase activity in cultured Band 2 cells from 25- to 40-day-old rats

3β-HSD activity of untreated Band 2 cells from 25-day-old rats was 0.104 ± 0.023 nmol

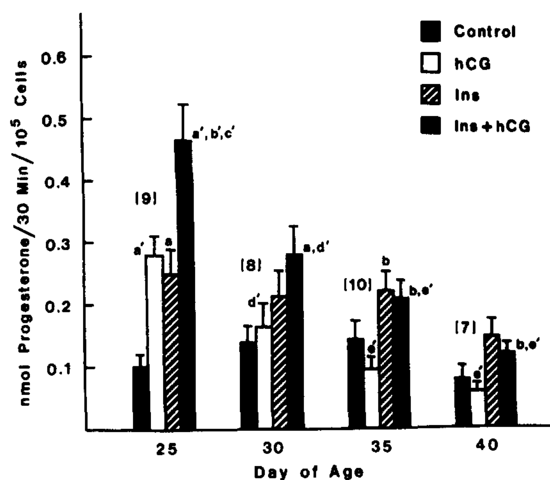

Fig. 1. Change in responsiveness with age of 3β-hydroxysteroid dehydrogenase-isomerase activity to hCG and/or insulin in cultured Band 2 cells. Assays were performed following 6 days of treatment. Numbers in parentheses represent total number of experiments, for each age group. a, a′: $P < 0.05$ and 0.01, respectively, when compared to control of the same age; b, b′: $P < 0.05$ and 0.01, respectively, when compared to hCG-treated group of the same age; c′: $P < 0.01$ when compared to insulin-treated group of the same age; d′: $P < 0.01$ when compared to the same treatment of the immediately younger age; e′: $P < 0.01$ when compared to the 25-day-old same treatment group.

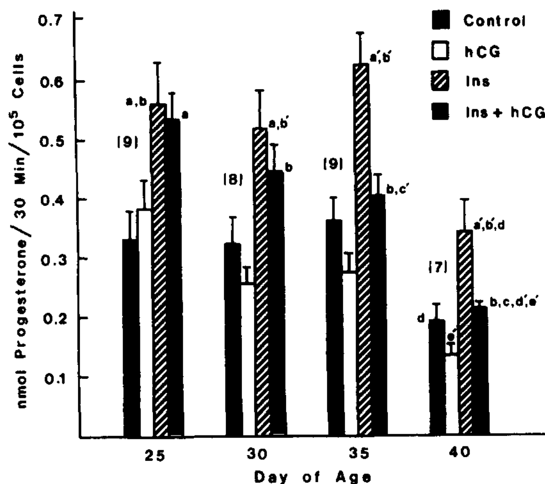

Fig. 2. Change in responsiveness with age of 3β-hydroxysteroid dehydrogenase-isomerase activity to hCG and/or insulin in cultured Band 3 cells. Assays were performed following 6 days of treatment. Numbers in parentheses represent total number of experiments, for each age group. a, a′: $P < 0.05$ and 0.01, respectively, when compared to control of the same age; b, b′: $P < 0.05$ and 0.01, respectively, when compared to hCG-treated group of the same age; c, c′: $P < 0.05$ and 0.01, respectively, when compared to insulin-treated group of the same age; d, d′: $P < 0.05$ and 0.01, respectively, when compared to the same treatment of the immediately younger age: e′: $P < 0.01$ when compared to the 25-day-old same treatment group.

progesterone/30 min/10^5 cells (Fig. 1). Activity increased to 0.276 ± 0.036 ($P < 0.01$) and 0.247 ± 0.043 nmol ($P < 0.05$) in response to 10 mIU/ml hCG or 2 μg/ml insulin, respectively. Treatment with hCG plus insulin increased activity further to 0.465 ± 0.058 nmol ($P < 0.01$) when compared to control, hCG- or insulin-treated groups. After day 25 hCG alone no longer stimulated 3β-HSD, and the response to hCG of Band 2 cells from 30-, 35- or 40-day-old rats declined when compared to the response in 25-day-old animals ($P < 0.05$). In Band 2 cells of animals older than 25 days of age, the increase in 3β-HSD with insulin was no longer statistically significant. Similarly, the overall response to hCG plus insulin declined when compared to the increase in 25-day-old rats ($P < 0.01$).

Effect of hCG and/or insulin on 3β-hydroxysteroid dehydrogenase-isomerase activity in cultured Band 3 cells from 25- to 40-day-old rats

In cultured Band 3 cells from 25-day-old rats 3β-HSD activity of untreated cells was 0.333 ± 0.057 nmol progesterone/30 min/10^5 cells (Fig. 2), which is about 3.2-fold higher than Band 2 control of the same age. Activity of Band 3 control cells from 30- and 35-day-old rats were similar; however, control activity of 40-day-old rats declined to 0.191 ± 0.033 nmol ($P < 0.05$). HCG did not increase 3β-HSD activity in Band 3 cells at any age. Insulin increased 3β-HSD activity when compared to control or hCG-treated group of each age. Treatment of Band 3 cells from 25-day-old rats with hCG plus insulin increased 3β-HSD activity when compared to control ($P < 0.05$); but not when compared to cells treated with insulin alone. In Band 3 cells from older rats hCG plus insulin did not increase 3β-HSD activity above control of the same age, but levels were higher than activity of cells treated with hCG alone. In Band 3 cells from 35- and 40-day-old animals hCG plus insulin actually decreased 3β-HSD activity when compared to cells treated with insulin alone ($P < 0.01$ and 0.05, respectively).

To ascertain whether the increase in 3β-HSD activity in Band 2 cells by hCG and/or insulin was due to cellular replication, DNA content of each well was determined in Band 2 and Band 3 cells from 25-day-old rats. Neither hCG and/or insulin increased DNA levels in Band 2 or Band 3 cells (Table 1).

Table 1. Effect of hCG and/or insulin on DNA levels of cultured Band 2 or Band 3 cells

Band	Treatment	μg DNA/10^5 cells
B_2	Control	2.64 ± 0.02
	Insulin	2.73 ± 0.10
	hCG	2.65 ± 0.05
	Insulin + hCG	2.73 ± 0.16
B_3	Control	2.36 ± 0.02
	Insulin	2.43 ± 0.10
	hCG	2.28 ± 0.10
	Insulin + hCG	2.10 ± 0.13

Each value represents the mean ± SEM of 3 separate experiments, each performed in duplicate. Assays were performed after 6 days of treatment. Insulin concentration was 2 μg/ml; hCG concentration was 10 mIU/ml.

Effect of hCG and/or insulin on histochemical staining for 3β-hydroxysteroid dehydrogenase in cultured Band 2 and Band 3 cells from 25-day-old rats

To determine whether increased 3β-HSD by hCG and/or insulin was due to precursor cell conversion to Leydig cells, we examined the effect of 6 days of treatment on the number of cells staining positive for 3β-HSD. Because it was difficult to identify unstained cells, we expressed our results as the total number of positive staining cells in 10 random fields rather than a percentage of total cells. Specific positive staining was identified by comparing with cells incubated without substrate. In untreated Band 2 cells we identified 50 ± 4 positive staining cells (Fig. 3), which increased to 212 ± 14 ($P < 0.01$) following hCG treatment. The modest increase with insulin was not statistically significant; however, hCG plus insulin increased the number of positive cells to 292 ± 28 which was higher than control ($P < 0.01$) and hCG-treated cells ($P < 0.01$).

There were 300 ± 50 positive staining cells in untreated Band 3 cells. Thus, the Band 3 vs Band 2 staining ratio of untreated cultured cells was 6, which closely approximated the ratio of 6.8 for freshly isolated cells. The number of positive staining cells was not altered by hCG or insulin, but increased to 495 ± 52 ($P < 0.05$) with hCG plus insulin when compared to control.

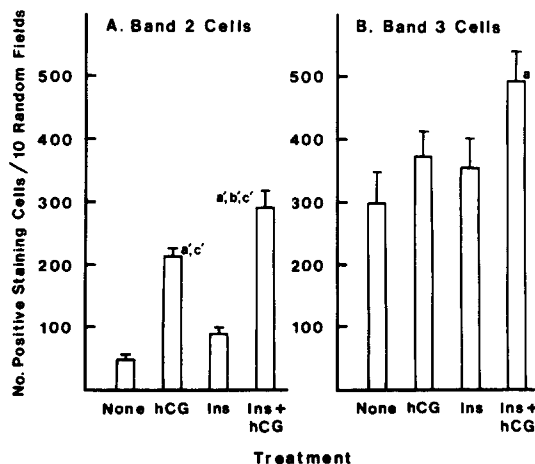

Fig. 3. Effect of hCG and/or insulin on histochemical staining for 3β-hydroxysteroid dehydrogenase in cultured Band 2 and Band 3 cells from 25-day-old rats. Treatment period was 6 days. Results for Band 2 and Band 3 cells represent the mean ± SEM of 8 and 6 separate experiments, respectively. a, a′: $P < 0.05$ and 0.01, respectively, when compared to the appropriate control; b′: $P < 0.01$ when compared to the appropriate hCG-treated group; c′: $P < 0.01$ when compared to the appropriate insulin-treated group. Magnification = 400 ×.

DISCUSSION

Previous studies demonstrated that 3β-HSD activity is limited to Leydig cells of testes [15, 19]. Therefore, we monitored this enzyme to assess Leydig cell responses to hCG and/or insulin between days 25 and 40 of maturation. Also, because only viable cells attach to culture dishes [20], Band 2 and Band 3 cells were cultured to eliminate cell fragments. The present studies demonstrate the viable Leydig cells localize in Band 2 from 25- to 40-day-old rats. Previous studies which identified damaged Leydig cells in Band 2 of metrizamide or Percoll gradients used adult animals [8, 9]. It is possible that Leydig cells from older animals are more susceptible to damage by these isolation procedures.

The Band 3 vs Band 2 3β-HSD staining ratios for freshly isolated and cultured cells from 25-day-old rats were 6.8 and 6, respectively. However, the 3β-HSD activity ratio (Band 3 vs Band 2 cells from 25-day-old rats) was 3.2. This suggests that histochemical staining for 3β-HSD does not detect all enzyme activity in Band 2 and misses Leydig cell precursors. The limitation of staining for 3β-HSD to identified Leydig cells was expressed previously [20].

It is not clear whether, *in situ*, the conversion of precusor cells localizing in Band 2 to "mature" Leydig cells which then localize in Band 3 is completed by about day 30 of maturation as suggested by the present results. The changing milieu of secreted androgens occurring between days 25–40 of maturation (due to elevation and later decline in 5α-reductase activity) [21] or of various regulatory factors may inhibit this conversion under culture conditions in animals around 30 days of age and older. Leydig cell destruction in adult rats by treatment with ethane dimethyl sulfonate is followed

by repopulation [22]; thus, precursor cells probably exist in adult testes. Also, an indeterminate connective tissue cell type which exhibited limited hCG-binding capacity and was thought to represent Leydig cell precursors was identified in Band 2 of adult animals [11]. Nevertheless, our data suggest that precursor cells localizing in Band 2 are not converted to "adult" Leydig cells under the present culture conditions after about 30 days of age. Although hCG converted precusor cells in Band 2 of 25-day-old rats to Leydig cells, insulin was more effective in increasing 3β-HSD activity in existing Leydig cells. Insulin was reported to increase testicular 3β-HSD activity when administered to intact or hypophysectomized diabetic rats [23].

The age dependency of 3β-HSD response to hCG has been reported previously. Thus, testicular 3β-HSD activity from 20- and 22-day-old rats was more responsive to hCG than testes of mature rats [24, 25]. Also, it was reported that *in vivo* administration of hCG to intact 20-day-old rats increased the number of positive 3β-HSD staining cells in testes [25]. This enhanced sensitivity in immature rats may be due to precursor cell conversion to Leydig cells.

There have been two published studies on the effect of hCG or cAMP on 3β-HSD in cultured rodent interstitial or Leydig cells. One study demonstrated that hCG increased 3β-HSD activity on cultured adult rat interstitial cells if testosterone synthesis was blocked by adding a 17α-hydroxylase inhibitor or an antiandrogen [26]. The other study showed that cAMP had no effect on 3β-HSD in cultured adult mouse Leydig cells [27]. Under the present conditions hCG had no effect on 3β-HSD in Leydig cells of Band 2 and Band 3 in animals 30 days of age and older. Although the present studies did not address the question of Leydig cell heterogeneity. The similarity in the overall direction of response to hCG and/or insulin of 3β-HSD in Band 2 and cells from 35- and 40-day-old rats suggest that these Leydig cells are similar.

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