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Enhanced Stimulation of 5α-Reductase Activity in Cultured Leydig Cell Precursors by Human Chorionic Gonadotropin

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Previous studies have demonstrated that the increase in number of Leydig cells during prepubertal maturation results, in part, from the differentiation of mesenchymal precursors between the second and fourth week of postnatal life. After conversion to immature Leydig cells, they actively synthesize testosterone, but this androgen does not accumulate because high 5α-reductase activity rapidly converts testosterone to 5α-reduced metabolites. The present studies examined whether the conversion of precursor cells to immature Leydig cells *in vitro* by human chorionic gonadotropin (hCG), as characterized by progressive increases in testosterone formation and 5-ene-3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) activity, is associated similarly with an enhanced stimulation of 5α-reductase activity. We also evaluated whether this conversion occurs following blockade of dihydrotestosterone (DHT) formation by the inclusion of a 5α-reductase inhibitor during the entire treatment period. Precursor cells were isolated from immature rats using a multi-step procedure normally used to isolate highly purified Leydig cells from adult or immature rats. These cells localize in a region of lower density on Percoll gradients than Leydig cells. Although the acute (3 h) response to hCG with respect to testosterone formation, and basal 3β-HSD and 5α-reductase activities on day 1 of culture were much higher in purified Leydig cells than precursor cells from immature rats, the response of each parameter to chronic (6-day) treatment with hCG was much greater in precursor cells. Furthermore, the conversion of precursor cells to immature Leydig cells occurred in the presence of a 5α-reductase inhibitor during the entire treatment period, suggesting that this conversion occurs in the absence of DHT. These results demonstrate for the first time that in addition to increased testosterone biosynthesis and 3β-HSD activity, the conversion of precursor cells to immature Leydig cells, *in vitro*, in response to chronic hCG treatment, involves enhanced 5α-reductase activity.

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INTRODUCTION

During fetal development and progressing through sexual maturation, rat Leydig cells exhibit three major stages of differentiation and change in functional activity [1]. Around day 15–16 of gestation, fetal Leydig cells appear and begin to secrete testosterone which functions to stabilize male reproductive structures [2]. Following birth fetal Leydig cells regress, and this is associated with a corresponding decline in circulating testosterone levels [3], although it has been reported that fetal Leydig cells actually persist in adult animals [4]. Around 14–28 days following birth, a second generation of Leydig cells appear, mainly through differentiation from mesenchymal precursor cells localized within the interstitium [5]. Following their appearance and to approx. 40 days of age, these immature Leydig cells actively synthesize testosterone; however, testosterone does not accumulate and is not secreted because high testicular 5α-reductase activity rapidly converts this androgen to 5α-reduced metabolites [6, 7]. Testosterone levels progressively increase after about 40 days of age because of the progressive decline in 5α-reductase activity [6, 7]. The third (adult) stage of Leydig cell differentiation occurs after about 60 days of age when 5α-reductase activity has declined to a very low level and testosterone becomes the primary androgen secreted [8].

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With earlier methods to prepare an enriched population of Leydig cells, collagenase dispersed interstitial cells from adult rats were centrifuged on Percoll density gradients alone [9]. This method yielded a Leydig cell fraction (~60–70% Leydig cells) which localized at a density of ~1.07 g/ml. Using the same isolation procedure for immature rats, we recently reported that Leydig cells of similar purity could be isolated from the same density region, but, in addition, Leydig cell precursors localized at a lower density region (density ~ 1.05 g/ml) [10]. These cultured precursor cells exhibited enhanced 5-ene-3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) activity following chronic treatment with human chorionic gonadotropin (hCG) [10]. More recently, a multi-step procedure has been described in which collagenase-dispersed interstitial cells are subjected to elutriation, and the cells retained at a set rotor and pump speed are centrifuged on a self-generated Percoll gradient [11]. In adult rats this procedure yields highly purified Leydig cells (>96% pure), which localize at a density of 1.068 g/ml and higher [11]. When this procedure for isolating purified Leydig cells was applied to immature rats, it was reported that in addition to highly purified immature Leydig cells, localizing at a density >1.070 g/ml, precursor cells could be isolated at a lower density region (between 1.064–1.070 g/ml) [12]. In that study the inclusion of dihydrotestosterone (DHT) at a high concentration enhanced the effect of LH in stimulating cultured precursor cell conversion to immature Leydig cells, as reflected by their increased capacity to synthesize testosterone [12].

In the present studies we re-evaluated the role of LH/hCG in stimulating the conversion of precursor cells to immature Leydig cells using the multi-step procedure to isolate both cell types. Basal and hCG-stimulated testosterone formation and 3β-HSD activity in both cultured precursor and purified Leydig cells from immature rats were first evaluated. We also examined whether the hCG-stimulated conversion of precursor cells to immature Leydig cells was associated with a significant increase in 5α-reductase activity, which is a prominent feature of immature Leydig cells.

MATERIALS AND METHODS

Animals

Immature Charles–Dawley rats were purchased from Charles-Rivers Labs (Raleigh, NC, U.S.A). Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. Animals were 24–25 days of age at the time of sacrifice. They were first rendered unconscious in a CO_2-saturated chamber, then killed by decapitation between 0800–0900 h. These procedures were approved by the local animal studies committee. Testes were excised and set at 4°C prior to dissociation with collagenase.

Reagents

Collagenase (Type 1), hCG, penicillin G, streptomycin, bovine serum albumin (BSA, RIA grade) and *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (Hepes) were from Sigma Chemical Co., (St Louis, MO, U.S.A.). Instant thin-layer chromatography sheets (ITLC) were from Gelman Sciences (Ann Arbor, MI, U.S.A.). The 5α-reductase inhibitor, 4-methyl-4-aza-3-oxo-5α-pregnan-20(s)-carboxylate (4-MAPC), was kindly provided by Merck, Sharp and Dohme, (Rahway, NJ, U.S.A.). Reagent grade organic solvents were from Fisher Scientific Co. (Atlanta, GA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (F12), and $NaHCO_3$ were from GIBCO BRL (Grand Island, NY, U.S.A.). Percoll was from Pharmacia Co., (Piscataway, NJ, U.S.A.). [1, 2, 6, 7,^{3}H]testosterone (99 Ci/mmol), [7-^{3}H] testosterone (25 Ci/mmol), [4-^{14}C]DHT (58 mCi/mmol), [7-^{3}H]pregnenolone (23 Ci/mmol) and [4-^{14}C]progesterone were from Dupont (NEN) Co., (Boston, MA, U.S.A.). 5α-[1α,2α(*n*)-^{3}H]Androstane-3α,17β-diol (3α-diol, 40 Ci/mmol) and 5α-[1α,2α(*n*)-^{3}H]androstane-3β;17β-diol (3β-diol, 40 Ci/mmol) were from Amersham Corp., (Arlington Heights, IL, U.S.A.). Unlabeled steroids were from Steraloids, (Wilton, NH, U.S.A.).

Isolation of interstitial cell fractions

Collagenase-dispersed interstitial cells were fractionated by elutriation and Percoll gradient centrifugation of cells retained at a pump setting and rotor speed of 16 ml/min and 2000 rpm, respectively, as described previously [11] but with modifications [13]. For our initial experiments the pump speed was first set at 8 ml/min and later increased to 16 ml/min, and cells eluting at both settings were saved. In later experiments, the pump speed was increased immediately to 16 ml/min, and the eluted cells were saved. The cells retained at this pump setting and a rotor speed of 2000 rpm were centrifuged on a 60% self-generating Percoll gradient. The cells localizing at a density of 1.068 g/ml or higher were saved as the purified Leydig cell (P2) fraction [11]. They represented 96 ± 1% Leydig cells based on histochemical staining for 3β-HSD [14]. Cells localizing at a density below 1.068 g/ml were identified as the P1 fraction [11]. In later experiments the P1 fraction was divided into two separate subfractions. The P1a subfraction represented cells localizing at a density <1.049 g/ml, while the P1b subfraction represented heavier cells localizing between densities of 1.049 and 1.068 g/ml. When we estimated the percentage of Leydig cells localizing in these fractions, the 8 and 8–16 ml/min (16 ml/min) elutriated fractions and P1 fraction contained 5.1 ± 0.8, 3.7 ± 0.6 and 5.0 ± 1.0% Leydig cells, respectively, based on positive staining for 3β-HSD, while the P1a and P1b subfractions contained 3.0 ± 0.6 and 13.5 ± 1.9% Leydig cells, respectively.

Table 1. Localization of 3β-HSD and 5α-reductase activities of interstitial cells fractionated by elutriation and Percoll gradient centrifugation

Enzyme activity	nmol/30 min/10^5 cells			
	8 ml/min	16 ml/min	P1	P2
3β-HSD	0.018 ± 0.001	0.008 ± 0.002	0.061 ± 0.002	0.397 ± 0.001
5α-Reductase	0.024 ± 0.002	0.008 ± 0.002	0.020 ± 0.001	0.185 ± 0.23

Enzyme activities were determined on day 1 of culture. Each value represents the mean ± SEM of three separate determinations from a single experiment and are representative of three separate studies.

Culture of interstitial cell fractions

The cellular fractions isolated by elutriation and Percoll gradient centrifugation of collagenase-dispersed interstitial cells were cultured at a concentration of 1×10^5/ml of DMEM-F12 (1:1 mixture) medium containing 15 mM Hepes buffer, pH 7.4, 1.2 g/l $NaHCO_3$, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.1% BSA as described previously [13]. Twenty four hours after plating cells were gently flushed with the original culture medium, and fresh medium was added to remove unattached cells and cellular debris. Treatment was initiated and continued for varying periods of time as described in the legend for each experiment. For extended cultures, fresh media were added and cells retreated every other day of culture.

3β-HSD and 5α-reductase assays on cultured cells and quantitation of testosterone

3β-HSD activity in cultured cells was determined by monitoring the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone as described previously [15]. 5α-Reductase activity in cultured cells was determined by monitoring the conversion of [^{3}H]testosterone to [^{3}H]DHT and [^{3}H]3α-diol and [^{3}H]3β-diol as described previously [16]. Testosterone released into the culture medium was quantitated by radioimmunoassay as described previously [17].

RESULTS

Localization of 3β-HSD and 5α-reductase activities in cultured interstitial cell fractions

We first established the localization of 3β-HSD and 5α-reductase activities in various fractions isolated using the multi-step procedure to isolate Leydig cells. 3β-HSD activity on day 1 of culture in the P2 fraction, which contains ~96% Leydig cells, was 0.397 ± 0.001 nmol progesterone/30 min/10^5 cells on day 1 of culture (Table 1). This activity was ~7-fold higher than the activity in the P1 fraction. 3β-HSD activity in the 8 and 8–16 ml/min elutriated fractions were even lower than the activity in the P1 fraction. 5α-Reductase activity in each fraction was similar to the pattern exhibited by 3β-HSD, with the highest activity (0.185 ± 0.023 nmol DHT + 3α + 3β-diol/30 min/10^5 cells) in P2 cells on day 1 of culture. This activity was 8- to 9-fold higher than the activity in the 8 and 8–16 ml/min elutriated fractions and in P1 cells.

Basal and acute hCG-stimulated testosterone formation in cultured interstitial cell fractions

Basal and 10 mIU/ml hCG-stimulated testosterone released into the medium of interstitial cell fractions were evaluated following an acute (3 h) exposure period on day 1 of culture. Cells were treated with 0.5 μM 4-MAPC during the treatment period to block 5α-reductase activity and prevent testosterone metabolism. In preliminary studies, this concentrtion of 4-MAPC completely blocked 5α-reductase activity in cultured Leydig cells from immature rats.

Basal testosterone formation was 0.041 ± 0.008 ng/3 h/10^5 cells or less in all fractions (Table 2). Testosterone levels increased in each fraction in response to hCG; however, the highest response was observed in P2 cells, which was more than 9-fold higher than testosterone formation by cultured P1 cells. These studies demonstrate that testosterone response to acute hCG stimulation in each fraction directly correlates with the percentage of Leydig cells localizing in that fraction.

Basal and chronic hCG-stimulated testosterone formation in cultured interstitial cell subfractions

Testosterone levels in control cells eluting at 8 ml/min and between 8–16 ml/min were undetectable during the entire 6-day treatment period [Fig. 1 (A, B, and C)]. Treatment with hCG increased testosterone, but levels were below 2 and 1 ng/ml for cells eluted

Table 2. Basal and acute testosterone response to hCG of cultured interstitial cell fractions isolated during multi-step purification of Leydig cells

Fraction	ng Testosterone/3h/10^5 cells	
	Control	+hCG
8 ml/min	0.035 ± 0.026	0.266 ± 0.093
16 ml/min	0.011 ± 0.007	0.054 ± 0.026
P1	0.015 ± 0.005	0.370 ± 0.152
P2	0.041 ± 0.008	3.407 ± 0.698

Media were changed on day 1 of culture and fresh media added. Cells were treated without or with 10 mIU/ml hCG for 3 h following addition of 0.5 μM 4-MAPC to block testosterone metabolism to 5α-reduced metabolites. Testosterone released into the medium was quantitated by radioimmunoassay. Results are the mean ± SEM of three separate experiments.

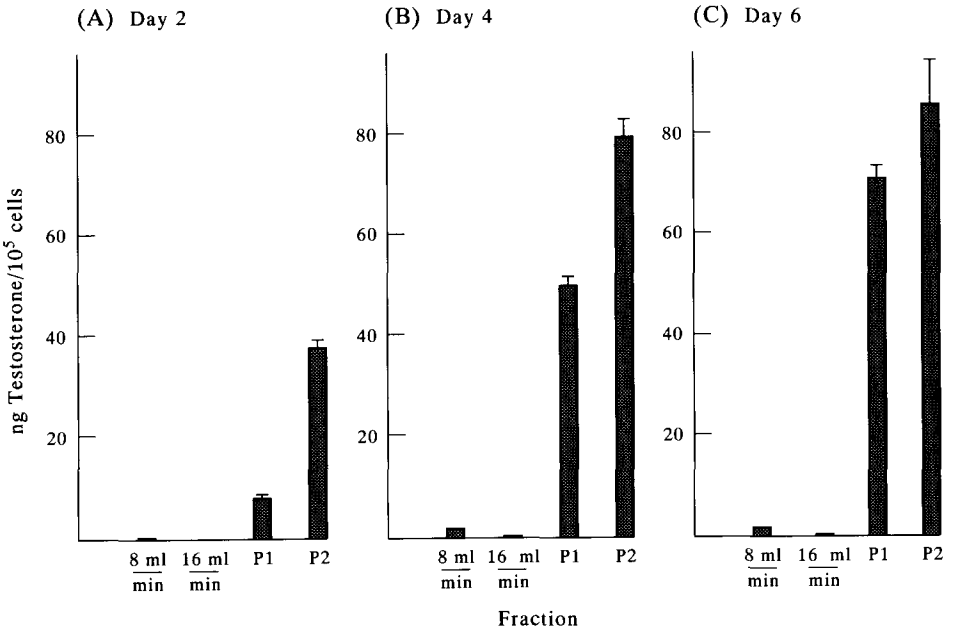

Fig. 1. Basal and hCG-stimulated testosterone formation in cultured interstitial cell fractions from immature rat testes. Cells were cultured in the presence of 0.5 μM 4-MAPC to block testosterone metabolism. Cells were treated with 10 mIU/ml hCG on days 1, 3, and 5 of culture. Testosterone released into the medium was quantitated by radioimmunoassay. Each value represents the mean ± SEM of three separate culture wells from a single experiment, and the results are representative of three separate experiments. Open bars = untreated controls of each fraction (values were too low to visualize using the above scale); solid bars = + hCG.

at 8 ml/min and between 8–16 ml/min, respectively, during the entire treatment period. Testosterone levels were <0.1 ng/10^5 cells in control P1 cells following 2 days of culture [Fig. 1, (A)] but were undetectable during the later days of culture [Fig. 1(B and C)]. In response to hCG, testosterone level was 7.5 ± 0.5 ng/10^5 cells following 2 days of treatment; however, levels increased to 50.3 ± 1.3 and 71.6 ± 2.9 ng/10^5 cells following 4 and 6 days of treatment, respectively. The latter was nearly 10-fold higher than cells treated for 2 days with hCG. The testosterone level in control P2 cells on day 2 was < 0.2 ng/10^5 cells, and levels remained below 0.3 ng on days 4 and 6. In response to hCG, the testosterone level on day 2 of treatment was 37.9 ± 1.1 ng, and levels increased to 80.1 ± 3.2 and 86.2 ± 10.4 ng on days 4 and 6 of treatment, respectively. The latter was approx. 2-fold higher than testosterone levels of P2 cells treated for 2 days with hCG. These results and those from the acute response to hCG (Table 2) demonstrate that although purified immature Leydig cells produce much higher levels of testosterone following acute (3 h) exposure to hCG, cultured P1 cells exhibit a much greater progressive increase in testosterone formation following extended exposure to hCG.

Basal and hCG-stimulated testosterone in cultured P1 subfractions

The preceding study demonstrated that although hCG-stimulated testosterone was significantly higher in P2 cells following 2 days of treatment, testosterone levels of P1 cells approached those of P2 cells as the treatment period increased to 4 and 6 days. In the previous studies, we collected all of the cells localizing at a density below 1.068 g/ml; however, cells within this area of Percoll gradient actually localize in two separate regions. One is a narrow band of cells localizing at a density below 1.049 g/ml (P1a) and the other is a broader band localizing between densities of 1.049 and 1.068 g/ml (P1b). These cells were separated and cultured in the presence of 0.5 μM 4-MAPC. Basal and hCG-stimulated testosterone levels were determined following 2 to 6 days of treatment to determine whether the more responsive cells could be localized within a more defined region. Untreated P1a and P1b cells produced very little testosterone during the entire observation period (all values were < 0.5 ng/10^5 cells) (Fig. 2). In response to hCG, P1a cells produced 8.1 ± 0.3 ng/10^5 cells following 2 days of treatment. Testosterone increased further to 31.7 ± 3.3 and 35.2 ± 5.0 ng following 4 and 6 days of treatment, respectively. P1b cells produced 15.8 ± 0.8 ng testosterone in response to hCG following 2 days of hCG treatment, and levels increased progressively to 69.8 ± 4.8 and 107.0 ± 6.9 ng following 4 and 6 days of treatment, respectively. These results suggest that the more responsive cells are concentrated in the P1b subfraction, but are not exclusive to this fraction.

Basal and hCG-stimulated 3β-HSD activity in P1b and P2 fractions

The previous study demonstrated that the P1b subfraction exhibited greater responsiveness to chronic hCG treatment with respect to testosterone formation.

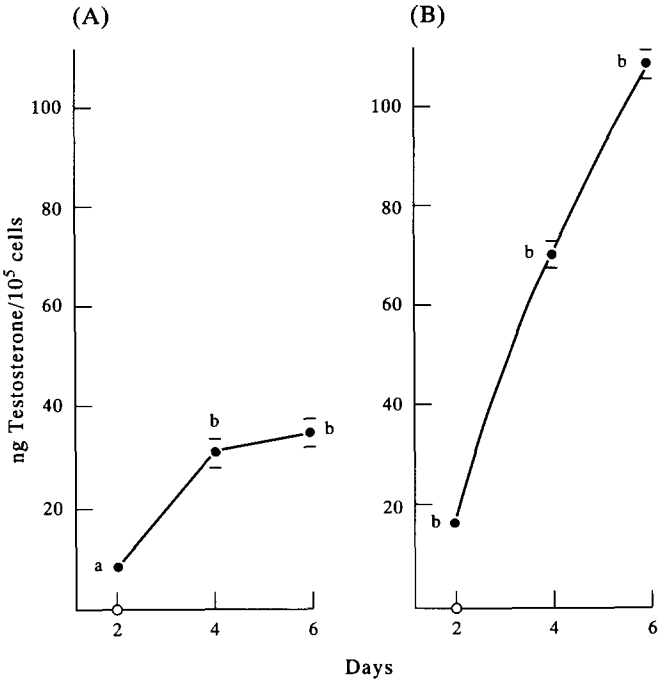

Fig. 2. Basal and hCG-stimulated testosterone formation in cultured P1 subfractions from immature rat testes. Cells were cultured in the presence of 0.5 μM 4-MAPC to block testosterone metabolism. Cells were treated with 10 mIU/ml hCG on days 1, 3 and 5 of culture. Testosterone released into the medium was quantitated by radioimmunoassay. Each value represents the mean ± SEM of three separate culture wells from a single experiment, and the results are representative of three separate experiments. Open circles = controls; solid circles = + hCG. (A) P1a fraction; (B) P1b fraction. [a] $P < 0.05$ when compared to day 0 control; [b] $P < 0.01$ when compared to day 0 control or controls of same period of treament.

We next compared basal and hCG-stimulated 3β-HSD activity of P1b and P2 cells following 0 to 6 days of treatment. Basal 3β-HSD activity of P1b cells on day 0 of treatment was 0.038 ± 0.002 nmol/30 min/10^5 cells [Fig. 3(A)]. Activity did not decline during the next 6 days of culture. In contrast, treatment with hCG increased 3β-HSD activity to 0.061 ± 0.001 ($P < 0.05$), 0.151 ± 0.003 ($P < 0.01$) and 0.267 ± 0.015 nmol following 2, 4, and 6 days of treatment, respectively, when compared to day 0 control. Activity in cells treated for 6 days with hCG was 6- to 7-fold higher than day 0 or 6-day controls. Both control and hCG-treated cells received 0.5 μM 4-MAPC to block testosterone metabolism, so that these changes occurred in the absence of DHT. In a previous study using precursor cells isolated from immature rats on Percoll density gradients alone, we showed that this increase in 3β-HSD activity was, in part, due to an increase in positive 3β-HSD staining cells in response to hCG [10].

Basal 3β-HSD activity of P2 cells on day 0 was 0.567 ± 0.029 nmol/30 min/10^5 cells [Fig. 3(B)]. Activity did not increase in these control cells during the entire 6-day treatment period. In contrast to P1b cells, hCG did not increase 3β-HSD activity of P2 cells during the entire 6-day treatment period.

Basal and hCG-stimulated 5α-reductase activity in P1b and P2 cells

The previous study demonstrated that 3β-HSD activity in P1b cells exhibited much greater responsiveness to hCG than purified Leydig cells (P2 cells). In the present study, we evaluated whether 5α-reductase in P1b cells exhibited a similar sensitivity to hCG. 5α-reductase activity in P1b cells on day 0 was 0.014 ± 0.002 nmol DHT + 3α- + β-diol/30 min/10^5 cells [Fig. 4(A)]. Activity declined progressively during the following 6 days of culture to 0.002 ± 0.001 nmol. Treatment with hCG increased activity to 0.032 ± 0.002 ($P < 0.01$), 0.053 ± 0.001 ($P < 0.01$) and 0.057 ± 0.001 nmol ($P < 0.01$), respectively, when compared to day 0 control or to controls of the same respective period of treatment. Following 6 days of treatment, 5α-reductase activity increased more than 4-fold above day 0 control. 5α-Reductase activity in P2 cells on day 0 of treatment was 0.186 ± 0.008 nmol DHT + 3α- + 3β-diol/30 min/10^5 cells [Fig. 4(B)]. Activity declined progressively during the following 6 days of culture, and was 0.030 ± 0.009 nmol on day 6. In response to hCG, 5α-reductase activity was higher at each period of treatment when compared to controls of the same respective time of treatment (all $P < 0.01$); however, when compared to day 0 control, activity increased only about 30 and 27% following 4 and 6 days of treatment, respectively.

DISCUSSION

The present studies demonstrate that cultured P1b cells from immature rat testes, which localize between densities of 1.049–1.068 g/ml on Percoll gradients, exhibit a greater net response to extended hCG treatment with respect to increases in testosterone formation, 3β-HSD and 5α-reductase activities than purified immature Leydig cells (P2 cells), although the latter cells exhibit significantly higher basal activities and hCG-stimulated testosterone levels following short-term treatment. We attribute this enhanced response to the presence of precursor cells in this fraction, which are converted to immature Leydig cells in response to chronic hCG treatment. The lower net response to chronic hCG in purified immature Leydig cells may be because testosterone synthesizing capacity and 3β-HSD and 5α-reductase activities are near maximal levels and cannot be further stimulated, appreciably, by hCG under the present conditions.

The present results confirm a recent study which reported that precursor cells localized in a lower density region of Percoll gradients (between densitites of 1.064–1.070 g/ml), when collagenase-dispersed interstitial cells from immature rats were elutriated and centrifuged on self-generated 55% Percoll gradients to isolate highly purified immature Leydig cells [12]. This density range is more restrictive, but within the range of the present study. The present results also confirm

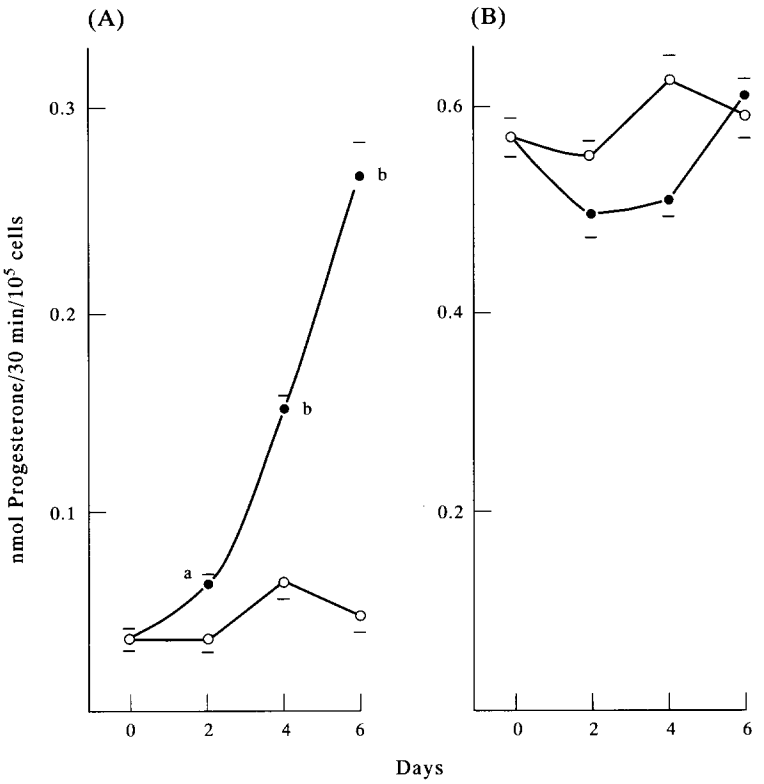

Fig. 3. Basal and hCG-stimulated 3β-HSD activity of cultured Leydig cell precursors and purified Leydig cells from immature rats. Cells were cultured in the presence of 0.5 μM 4-MAPC to block testosterone metabolism. Treated cells received 10 mIU/ml hCG on days 1, 3, and 5 of culture. 3β-HSD activity of intact cells was determined following 2, 4, or 6 days of treatment. These results represent the mean ± SEM of three separate culture wells from a single experiment, and they are representative of three separate experiments. Open circles = controls; solid circles = + hCG. (A) Leydig cell precursors; (B) purified Leydig cells. [a] $P < 0.05$ when compared to day 0 control or day 2 control; [b] $P < 0.01$ when compared to day 0 control or controls of the same period of treatment.

evidence for precursor cells localizing in a lower density region of Percoll gradients (B2, ~1.05 g/ml) when collagenase-dispersed interstitial cells from immature rats were centrifuged on a 15–60% continuous Percoll gradient alone to isolate purified Leydig cells [10]. In addition, another study identified indeterminate connective tissue cells (probably representing precursor cells) in a lower density range of Percoll gradients (between 1.044–1.059 g/ml) when collagenase-dispersed interstitial cells from immature rats (21–25 days of age) were centrifuged on Percoll gradients alone [18]. These cells had the capacity to bind [^{125}I] hCG.

In the present study both testosterone formation and 3β-HSD activity increased progressively during the 6-day hCG treatment period. These increases occurred when cells were cultured with the 5α-reductase inhibitor, 4-MAPC, during the entire treatment period to prevent testosterone metabolism and, thus, to block DHT formation. Thus, in contrast to a previous study where both exogenous LH and DHT were required to increase the testosterone synthesizing capacity of precursor cells [12], the present changes occurred in response to hCG alone. Because the inclusion of 4-MAPC to cultured precursor cells results in the accumulation of testosterone [20], it is possible that testosterone functioned as the effective androgen rather than DHT in the present studies. This appears unlikely because a previous study demonstrated that high levels of testosterone had no effect on 5α-reductase activity in rat testes of immature hypophysectomized rats [19]. Therefore, under the present experimental conditions, it appears that LH/hCG alone can stimulate the conversion of precursor cells to immature Leydig cells.

Previous studies have demonstrated that immature Leydig cells originate, in part, from the differentiation of mesenchymal precursors during a limited period of maturation (between 14–28 days after birth) [5]. In support of these observations, we reported that the hCG-stimulated conversion of cultured precursor cells could not be demonstrated after about day 30 of maturation [10]. After precursor cells have differentiated into immature Leydig cells, *in vivo*, they express high 5α-reductase activity [6, 7]. In the present studies we demonstrated for the first time that the hCG-stimulated conversion of cultured precursor cells to immature Leydig cells is associated with a progressive increase in 5α-reductase activity, which is similar to the progressive increase in testosterone synthesizing capacity and 3β-HSD activity. This increase in 5α-reductase activity would preclude appreciable accumulation of testosterone in cells stimulated with LH/hCG. After precursor cells have been converted to immature

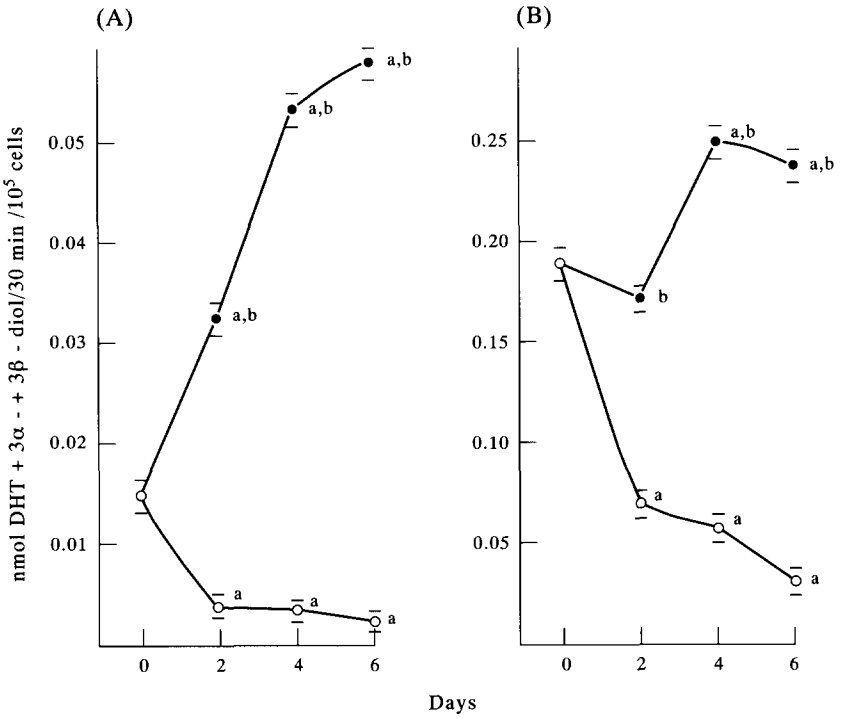

Fig. 4. Basal and hCG-stimulated 5α-reductase activity of cultured Leydig cell precursors and purified Leydig cells from immature rats. Treated cells received 10 mIU/ml hCG on days 1, 3, and 5 of culture. 5α-Reductase activity was determined on intact cells following 2, 4, and 6 days of treatment. Open circles = controls; solid circles = + hCG. (A) Leydig cells precursors; (B) purified Leydig cells. [a] $P < 0.01$ when compared to day 0 control; [b] $P < 0.01$ when compared to controls of same period of treatment.

Leydig cells (P2 cells in the present study), it appears that the main effect of LH/hCG is to maintain 5α-reductase activity at a high level characteristic of immature Leydig cells. It is still not clear what causes the selective decline in 5α-reductase activity in Leydig cells after about 40 days of age, although a recent study suggested that basic fibroblast growth factor may, in part, be involved [21].

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REFERENCES

1. Kuopio T., Tapanainen J., Pelliniemi L. J. and Huhtaniemi I.: Developmental stages of fetal-type Leydig cells in prepubertal rats. *Development* **107** (1989) 213–220.
2. Niemi M. and Ikonen M.: Steroid-3β-ol-dehydrogenase activity in foetal Leydig's cells. *Nature* **189** (1961) 592–593.
3. Tapanainen J., Kuopio T., Pelliniemi L. J. and Huhtaniemi I.: Rat testicular endogenous steroid and number of Leydig cells between the fetal period and sexual maturation. *Biol Reprod.* **31** (1984) 1027–1035.
4. Kerr J. B. and Knell C. M.: The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. *Development* **103** (1988) 535–544.
5. Hardy M. P., Zirkin B. R. and Ewing L. L.: Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. *Endocrinology* **124** (1989) 762–770.
6. Inano H., Hori Y. and Tamaoki B.-I.: Effect of age on testicular enzymes related to steroid bioconversion. *Ciba Found. Colloq. Endocr.* **16** (1967) 105–119.
7. Coffey J. C., French F. S. and Nayfeh S. N.: Metabolism of progesterone by rat testicuar homogenates. IV. Further studies of testosterone formation on immature testis *in vitro*. *Endocrinology* **89** (1971) 865–872.
8. Corpechot C., Baulieu E.-E. and Robel P.: Testosterone, dihydrotestosterone and androstanediols in plasma, testes, and prostates of rats during development. *Acta Endocr.* **96** (1981) 127–135.
9. Browning J. Y., D'Agata R. and Grotjan H. E. Jr: Isolation of purified rat Leydig cells using continuous Percoll gradients. *Endocrinology* **109** (1981) 667–669.
10. Murono E. P. and Washburn A. L.: Evidence that Leydig cell precursors localize in immature band two cells isolated on Percoll gradients. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 675–680.
11. Klinefelter B. R., Hall P. F. and Ewing L. L.: Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure *Biol. Reprod.* **36** (1987) 769–783.
12. Hardy M. P., Kelce W. R., Klinefelter G. R. and Ewing L. L.: Differentiation of Leydig cell precursors *in vitro:* a role for androgen. *Endocrinology* **127** (1990) 488–490.
13. Murono E. P., Washburn A. L., Goforth D. P. and Wu N.: Evidence for basic fibroblast growth factor receptors in cultured immature Leydig cells. *Molec. Cell Endocr.* **88** (1992) 39–45.
14. Wiebe J. P.: Steroidogenesis in rat Leydig cells: changes in activity of 5-ane and 5-ene 3β-hydroxysteroid dehydrogenases during sexual maturation. *Endocrinology* **98** (1976) 505–513.
15. Murono E. P. and Washburn A. L.: Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity in two distinct density Leydig cells from immature rats. Differences in responsiveness to human chorionic gonadotropin or 8-bromoadenosine 3′,5′-monophosphate. *Biochim. Biophys. Acta* **1091** (1991) 55–62.
16. Murono E. P. and Washburn A. L.: Regulation of 5α-reductase activity in cultured immature Leydig cells by human chorionic gonadotropin. *J. Steroid Biochem.* **35** (1990) 715–721.
17. Murono E. P., Lin T., Osterman J. and Nankin H. R.: The effects of cytochalasin B on testosterone synthesis by interstitial cells of rat testis. *Biochim. Biophys. Acta* **633** (1980) 228–236.
18. Risbridger G. P. and deKretser D. M.: Percoll-gradient separation of Leydig cells from postnatal rat testes. *J. Reprod. Fert.* **73** (1986) 331–338.
19. Nayfeh S. N., Coffey J. C., Hansson V. and French F. S.: Maturational changes in testicular steroidogenesis: hormonal regulation of 5α-reductase. *J. Steroid Biochem.* **6** (1975) 329–335.

20. Murono E. P. and Washburn A. L.: 5α-reductase activity regulates testosterone accumulation in two bands of immature cultured Leydig cells isolated on Percoll density gradients. *Acta Endocr.* **121** (1989) 538–544.

21. Murono E. P and Washburn A. L.: Fibroblast growth factor inhibits 5α-reductase activity in cultured immature Leydig cells. *Molec. Cell. Endocr.* **68** (1990) R19–23.