Maturational changes in steroidogenic enzyme activities metabolizing testosterone and dihydrotestosterone in two populations of testicular interstitial cells

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Abstract. The present study examined changes in steroidogenic enzyme activities which metabolize testosterone or dihydrotestosterone between days 21–73 of maturation in Band 2 and Band 3 cells isolated by centrifugation of rat testicular interstitial cells on metrizamide density gradients. 5α-reductase and 17β-hydroxysteroid dehydrogenase activities increased progressively in Band 2 and Band 3 cells between days 21–35 of maturation, then both enzyme activities declined to reach low levels in adult Band 2 and Band 3 cells. The significantly higher activities of both enzymes in Band 3, which contains a greater concentration of Leydig cells at each age, suggest their localization in Leydig cells. 5α-androstane-3α- and 3β-hydroxysteroid dehydrogenase activities increased in both Band 2 and Band 3 cells between days 21–50 of maturation and remained elevated; however, dihydrotestosterone was metabolized primarily to 5α-androstane-3α,17β-diol in Band 2 cells, while 5α-androstane-3β,17β-diol was the major metabolite of dihydrotestosterone in Band 3 cells. These studies suggest that testosterone accumulation during sexual maturation can be influenced by changing patterns of 5α-reductase and 17β-hydroxysteroid dehydrogenase activities which metabolize testosterone, and of 5α-androstane-3α- and 3β-hydroxysteroid dehydrogenase activities which metabolize dihydrotestosterone in both Band 2 and Band 3 cells.

Fetal Leydig cells appear around day 15-16 of gestation in rodents, and they begin to produce testosterone, which regulates differentiation of the male reproductive tract and accessory sexual organs (1). After birth fetal Leydig cells regress (2,3); however, between the second and third week of postnatal life, a second generation of Leydig cells appears and their numbers increase during pubertal maturation (4). The appearance of this 'adult' generation of Leydig cells correlates with the localization on density gradients of a second band of cells which bind [125I]hCG (5). Thus, around the third week of postnatal life, interstitial cells localize in at least two distinct bands on density gradients: a denser Band 3 (B₃) or population II and a less dense Band 2 (B₂) or population I (6). It has been reported that B₂ Leydig cells give rise to B₃ Leydig cells during sexual maturation (7). However, another study suggested that Leydig cells localizing in B₂ represented damaged cells, thereby implying a homogenous population of Leydig cells (8). More recently, the cells appearing damaged in B₂ were identified as indeterminant connective tissue cells, which may be precursors of adult Leydig cells (9).

During prepubertal maturation (~20–40 days of age) Leydig cells synthesize testosterone; however, this androgen does not accumulate because elevated 5α-reductase activity converts testosterone to dihydrotestosterone (DHT) (10), which in turn is metabolized to 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), and androsterone (11–13). Consequently, these 5α-reduced metabolites of testosterone represent the major androgens secreted by the immature testis (13, 14). Testosterone becomes the major androgen after ~40 days of age because 5α-reductase activity declines, resulting in less testosterone metabolism.
Because Leydig cells from ~20- to 40-day-old animals continue to differentiate with respect to steroidogenic competence, especially in regard to testosterone accumulation and 5α-reductase activity, they may not be adult in a functional sense.

The present study examined 5α-reductase activity in both B₂ and B₃ cells between days 21–73 of maturation because of the controversy over the functional significance of Leydig cells localizing in B₂ and B₃, and the key role of this enzyme in regulating testosterone accumulation in the maturing rat testis. It was reasoned that if B₂ Leydig cells functionally differ from those localizing in B₃, and if 5α-reductase activity is localized in Leydig cells, the activity profile of the enzyme in each band may differ. In addition, because testosterone accumulation could be influenced by other enzymes which metabolize testosterone or DHT, 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, which converts testosterone to androstenedione, and 5α-androstane-3α-hydroxysteroid dehydrogenase (5α-ane-3α-HSD) and 5α-androstane-3β-hydroxysteroid dehydrogenase (5α-ane-3β-HSD) activities which convert DHT to 3α- and 3β-diol, respectively were measured.

**Materials and Methods**

**Animals**

Sprague-Dawley rats were obtained from Zivic-Miller Laboratories, Zelienople, PA. They were maintained at 22°C with a controlled light cycle (12 h light and 12 h dark) and provided dry rat chow and tap water ad libitum. They were rendered unconscious with CO₂, then decapitated between 08.00 and 09.00 h. Testes were quickly removed and maintained at 4°C.

**Reagents**

Collagenase (Type I), bovine serum albumin, NAD⁺, etiocholanolone, nitro blue tetrazolium, and dimethyl sulfoxide were from Sigma Chemical Co, St. Louis, MO. Reagent grade organic solvents were from Fisher Scientific Co, Atlanta, GA. Medium 199 was from Grand Island Biological Co, Grand Island, NY. The labelled steroids [7-³H]testosterone (25 Ci/mmol), [4-¹⁴C]dihydrotestosterone (58 mCi/mmol) and [4-¹⁴C]androstenedione (52 mCi/mmol) were from New England Nuclear, Boston, MA. 5α-dihydro [1α,2α(n)-³H]testosterone (54 Ci/mmol), 5α[1α,2α(n)-¹⁴H]androstane-3α,17β-diol (40 Ci/mmol) and 5α[1α,2α(n)-¹⁴H]androstane-3β,17β-diol (40 Ci/mmol) were from Amersham Corp, Arlington Heights, IL. The purity of each labelled steroid was assessed by chromatography or recrystallization. Unlabelled androstenedione, testosterone, dihydrotestosterone, 5α-androstane-
3α,17β-diol and 5α-androstane-3β,17β-diol were from Steraloids, Wilton, New Hampshire. Each was recrystallized prior to use. Metrizamide was from Nyegaard & Co, Oslo, Norway.

Isolation of Band 2 and Band 3 cells

Testes were decapsulated and dispersed with 0.025% collagenase for 20 min as described previously (15). Interstitial cells were layered over a 20-ml, 0–32% continuous metrizamide gradient and centrifuged for 15 min at 3300 g to isolate B₂ and B₃ cells (16). Isolated B₂ and B₃ cells were washed 3 times with ~20 ml each of Medium 199 containing 0.1% bovine serum albumin (Med 199-BSA) and 14 mmol/l NaHCO₃.

Enzyme assays

5α-reductase activity was estimated by incubating intact B₂ (2–4 × 10⁴ cells/l) and B₃ cells (1–2 × 10⁴ cells/l) in the presence of [³H]testosterone (10 μmol/l, 0.5 μCi) for 30 min at 34°C in an atmosphere of 95% O₂:5% CO₂ in Med 199-BSA. In preliminary studies it was demonstrated that the reaction was linear during this interval and that this was a saturating substrate concentration. Reactions were terminated by boiling the samples for 5 min. Unlabelled testosterone, androstenedione, DHT, 3α-diol and 3β-diol (50 μg each) were added to serve as carriers and ~4000 cpm of [¹⁴C]DHT to estimate recovery. In separate incubation tubes ~2000 cpm each of [³H]3α-diol or [³H]3β-diol was added for recovery estimates. Samples were extracted with 5 vol of diethyl ether, the organic phase was dried under vacuum, and the residues were chromatographed on instant thin-layer chromatography sheets (ITLC-SA, Gelman, Ann Arbor, MI) using chloroform:methanol (99.0:1.0, vol/vol) as the solvent system. We did not attempt to separate 3α- and 3β-diol. 5α-reductase activity was estimated as (nmol DHT + 3α- and 3β-diol) · (30 min)⁻¹ · (10⁵ cells)⁻¹.

The procedure to estimate 17β-hydroxysteroid dehydrogenase activity was identical to that for 5α-reductase described above, except that 50 μg each of unlabelled testosterone and androstenedione and ~4000 cpm [¹⁴C]androstenedione was added after terminating the reaction, and androstenedione was isolated by ITLC.

Activities for 5α-3α-3β-HSD and 5α-3β-HSD were estimated as described previously (17). The products 3α- and 3β-diol were separated by ITLC with two ascents of benzene:ethanol (96:4, vol/vol) as the solvent system (18).

3β-hydroxysteroid dehydrogenase (β-HSD) histochemical staining to identify Leydig cells

Leydig cells were identified by histochemical staining for 3β-HSD using etiocholanolone as substrate (19). Nonspecific staining was determined by omitting etiocholanolone from the incubation mixture. Darkly staining cells were identified as Leydig cells.

Results

Maturational changes in 5α-reductase activity in B₂ and B₃ cells

5α-reductase activity in B₂ cells on day 21 was 0.017 nmol · (30 min)⁻¹ · (10⁵ cells)⁻¹. Activity progressively increased to peak at 0.147 ± 0.014 nmol on day 35, then decreased to 0.018 ± 0.008 nmol on day 53 and remained low through day 73 (Fig. 1, Panel A). 5α-reductase activity in B₃ cells was 0.305 nmol · (30 min)⁻¹ · (10⁵ cells)⁻¹ on day 21. Activity progressively increased to peak at 1.404 ± 0.083
Maturational changes in 5α-androstane-3α-hydroxysteroid dehydrogenase and 5α-androstane-3β-hydroxysteroid dehydrogenase activities in Band 2 cells. See legend to Fig. 1 for abbreviations and details on the preparation of cells and the number of determinations for each age. In Band 2 cells, 17β-HSD activity was not detected on day 21 (Fig. 2, Panel A). Activity was 0.010 nmol · (30 min)⁻¹ · (10⁶ cells)⁻¹ on day 24, which increased to peak at 0.148 ± 0.028 nmol on day 35. Activity declined to 0.020 ± 0.001 nmol on day 53, and remained low through day 73. In Band 3 cells, 17β-HSD activity on day 21 was 0.090 nmol, which increased to peak at 1.260 ± 0.126 on day 35 (Fig. 2, Panel B). Activity then declined, and was 0.131 ± 0.020 nmol on day 73. At each age, 17β-HSD activity was higher in Band 3 cells.

Maturational changes in 5α-ane-3α-HSD and 5α-ane-3β-HSD activities in Band 2 and Band 3 cells
In Band 2 cells, 5α-ane-3α-HSD activity on day 21 was 0.128 nmol · (10 min)⁻¹ · (10⁶ cells)⁻¹ (Fig. 3). Activity increased to 0.553 ± 0.056 nmol on day 39 and remained elevated through day 73. In Band 3 cells, 5α-ane-3β-HSD activity on day 21 was 0.025 nmol ·
(10 min)·(10³ cells)⁻¹ (Fig. 3). Activity increased to peak at 0.163 ± 0.015 nmol on day 35, and activity remained elevated through day 73. At each age DHT was converted primarily to 3α-diol in B₂ cells.

In B₃ cells 5α-ane-3β-HSD activity on day 21 was 0.246 nmol·(10 min)·(10³ cells)⁻¹ (Fig. 4). Activity increased thereafter and was 1.243 ± 0.272 nmol on day 63 and stabilized. In B₂ cells 5α-ane-3α-HSD activity on day 21 was 0.182 nmol·(10 min)·(10³ cells)⁻¹ (Fig. 4). Activity increased after day 30 to peak at 0.451 ± 0.039 nmol on day 43, and remained elevated through day 73. At each age DHT was converted mainly to 3β-diol in B₂ cells, and 5α-ane-3β-HSD activity in B₃ cells was higher than in B₂ cells.

Staining for 3β-HSD in B₂ and B₃ cells
Between days 21—27, 3—6% of B₂ cells stained positive for 3β-HSD (Fig. 5). On days 30, 32 and 35, the percentage of B₂ cells staining positive were 8, 16 and 12%, respectively. Thereafter, 4—6% of B₂ cells stained positive for 3β-HSD. On day 21, 12% of B₃ cells stained positive for 3β-HSD (Fig. 5). The percentage of positive staining cells increased to over 60% by day 30, and remained between 60—76% through day 73.

Discussion

The present results demonstrate that 5α-reductase and 17β-HSD activities increase between days 21—35 in both B₂ and B₃ cells, then decline to reach low levels in adult B₂ and B₃ cells. In both B₂ and B₃ cells 5α-ane-3α-HSD and 5α-ane-3β-HSD activities increased between days 21—50; however, 3α-diol was the major metabolite of DHT in B₂ cells, while 3β-diol was the primary metabolite of DHT in B₃ cells. After peaking, the activity of each enzyme metabolizing DHT remained elevated in adult B₂ and B₃ cells.

The developmental profile of 5α-reductase activity in both B₂ and B₃ cells in the present study closely follows the pattern previously reported in whole testes (10). The presence of elevated 5α-reductase activity in B₂ and B₃ cells of immature rats supports the observation that more 3α-diol than testosterone is secreted by immature B₂ and B₃ cells (7). The presence of higher 5α-reductase activity in B₃ cells suggests that the enzyme is localized in Leydig cells, because at each age B₃ cells had a greater percentage of Leydig cells in the present study. Although 5α-reductase activity has been identified in seminiferous tubules (20, 21), studies suggest that the enzyme is concentrated in interstitial tissue of immature rats (22, 23). Whether 5α-reductase activity is localized exclusively in Leydig cells remains to be determined. Previous studies identified 5α-reductase activity in lung macrophages (24) and skin fibroblasts (25), and macrophages and fibroblasts are found in interstitial tissue (5). Thus, more refined methods of cell separation are required to determine whether 5α-reductase activity is present in these cells.

The pattern of 17β-HSD activity observed in both B₂ and B₃ cells in the present study confirms the maturational profile reported previously on whole testis homogenates (26). Because 17-ketosteroid reductase activity, which converts androstenedione to testosterone, remains elevated in adult testes (26, 27), this suggests distinct enzymes. The higher specific activity of 17β-HSD in B₃ cells suggests that the enzyme is localized in Leydig cells; however, similar to the question of 5α-reductase localization, a more complete separation of cell types comprising B₃ and B₂ is necessary before we can be certain that the enzyme is limited to Leydig cells. If 17β-HSD and 5α-reductase are localized in Leydig cells, the similar activity profile in B₂ and B₃ demonstrates common characteristics of Leydig cells localizing in each band.

In a previous study on crude collagenase dispersed interstitial cell homogenates, 5α-ane-3β-HSD peaked between days 30—35 of maturation, then declined by over 70% by day 71 (19). The increase in 5α-ane-3β-HSD activity during early maturation is similar to the pattern observed with B₃ cells in the present study; however, in the present study, activity remained elevated after peaking. This discrepancy may be due to differences in the types of cells used (crude interstitial cells vs metrizamide gradient-isolated interstitial cells) or in the incubation conditions (Wiebe (19) used 12 000 × g supernatants and included cofactor, while we used intact cells without exogenous cofactor). The conversion of DHT mainly to 3β-diol by B₃ cells in the present study confirms previous reports on intact rat interstitial cells after day 20 of maturation which observed higher 3β-diol levels in cells incubated with [3H]DHT (28), and in adult intact B₃ cells (17).

It is demonstrated for the first time that 5α-ane-
3α-HSD activity is higher in B₂ cells. The increase in 5α-11-c-progesterone homogenates. IV. Further studies of testosterone formation in immature testes in vitro. Endocrinology 1971; 89:856-72.


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