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

Eisuke P. Murono

Medical Research Service, WJB Dorn Veterans’ Hospital and Departments of Medicine and Physiology, University of South Carolina School of Medicine, Columbia, South Carolina, USA

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 5α- 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)-3H]androstan-3α,17β-diol (40 Ci/mmol) and 5α[1α,2α(n)-3H]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-
3\sigma, 17\beta-diol and 5\alpha-androstane-3\beta, 17\beta-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 \times 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

5\alpha-reductase activity was estimated by incubating intact B₂ (2–4 \times 10⁶ cells/l) and B₃ cells (1–2 \times 10⁶ 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, 3\alpha-diol and 3\beta-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]3\alpha-diol or [³H]3\beta-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 3\alpha- and 3\beta-diol. 5\alpha-reductase activity was estimated as (nmol DHT + 3\alpha- and 3\beta-diol) \cdot (30\text{ min}) \cdot (10^9\text{ cells})^-1.

The procedure to estimate 17\beta-hydroxysteroid dehydrogenase activity was identical to that for 5\alpha-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 5\alpha-3\alpha-HSD and 5\alpha-3\beta-HSD were estimated as described previously (17). The products 3\alpha- and 3\beta-diol were separated by ITLC with two ascents of benzene:ethanol (96:4, vol/vol) as the solvent system (18).

5\beta-hydroxysteroid dehydrogenase (5\beta-HSD)

Histochemical staining to identify Leydig cells

Leydig cells were identified by histochemical staining for 5\beta-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 5\alpha-reductase activity in B₂ and B₃ cells

5\alpha-reductase activity in B₂ cells on day 21 was 0.017 nmol \cdot (30\text{ min})^-1 \cdot (10^5\text{ 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). 5\alpha-reductase activity in B₃ cells was 0.305 nmol \cdot (30\text{ min})^-1 \cdot (10^5\text{ 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.

nmol on day 35, then activity declined to 0.084 ± 0.007 nmol on day 53 and remained at this low level through day 73 (Fig. 1, Panel B). At each age a greater percentage of DHT was converted to 3α- and 3β-diol in B2 cells; however, total 5α-reductase was higher in B3 cells at each age.

Maturational changes in 17β-hydroxysteroid dehydrogenase activity in B2 and B3 cells

In B2 cells 17β-HSD activity was not detected on day 21 (Fig. 2, Panel A). Activity was 0.010 nmol·(30 min)⁻¹·(10⁵ 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 B3 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 B3 cells.

Maturational changes in 5α-ane-3α-HSD and 5α-ane-3β-HSD activities in B2 and B3 cells

In B2 cells 5α-ane-3α-HSD activity on day 21 was 0.128 nmol·(10 min)⁻¹·(10⁵ cells)⁻¹ (Fig. 3). Activity increased to 0.553 ± 0.056 nmol on day 39 and remained elevated through day 73. In B3 cells 5α-ane-3β-HSD activity on day 21 was 0.025 nmol·
(10 min)$^{-1} \cdot (10^5 \text{ cells})^{-1}$ (Fig. 3). Activity increased to peak at $0.163 \pm 0.015 \text{ nmol on day 35}$, and activity remained elevated through day 73. At each age DHT was converted primarily to $3\alpha$-dihydrotestosterone in $B_2$ cells.

In $B_2$ cells $5\alpha$-androstane-3$\beta$-HSD activity on day 21 was 0.246 nmol $\cdot (10 \text{ min})^{-1} \cdot (10^5 \text{ cells})^{-1}$ (Fig. 4). Activity increased thereafter and was $1.243 \pm 0.272 \text{ nmol on day 63}$ and stabilized. In $B_2$ cells $5\alpha$-androstane-3$\alpha$-HSD activity on day 21 was 0.182 nmol $\cdot (10 \text{ min})^{-1} \cdot (10^5 \text{ cells})^{-1}$ (Fig. 4). Activity increased after day 30 to peak at $0.451 \pm 0.039 \text{ nmol on day 43}$, and remained elevated through day 73. At each age DHT was converted mainly to $3\beta$-dihydrotestosterone in $B_2$ cells, and $5\alpha$-androstane-3$\beta$-HSD activity in $B_3$ cells was higher than in $B_2$ cells.

**Staining for $3\beta$-HSD in $B_2$ and $B_3$ cells**

Between days 21–27, 3–6% of $B_2$ cells stained positive for $3\beta$-HSD (Fig. 5). On days 30, 32 and 35, the percentage of $B_2$ cells staining positive were 8, 16 and 12%, respectively. Thereafter, 4–6% of $B_2$ cells stained positive for $3\beta$-HSD. On day 21, 12% of $B_3$ cells stained positive for $3\beta$-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 $5\alpha$-reductase and $17\beta$-HSD activities increase between days 21–35 in both $B_2$ and $B_3$ cells, then decline to reach low levels in adult $B_2$ and $B_3$ cells. In both $B_2$ and $B_3$ cells $5\alpha$-androstane-3$\alpha$-HSD and $5\alpha$-androstane-3$\beta$-HSD activities increased between days 21–50; however, $3\alpha$-dihydrotestosterone was the major metabolite of DHT in $B_2$ cells, while $3\beta$-dihydrotestosterone was the primary metabolite of DHT in $B_3$ cells. After peaking, the activity of each enzyme metabolizing DHT remained elevated in adult $B_2$ and $B_3$ cells.

The developmental profile of $5\alpha$-reductase activity in both $B_2$ and $B_3$ cells in the present study closely follows the pattern previously reported in whole testes (10). The presence of elevated $5\alpha$-reductase activity in $B_2$ and $B_3$ cells of immature rats suggests the observation that more $3\alpha$-dihydrotestosterone is secreted by immature $B_2$ and $B_3$ cells (7). The presence of higher $5\alpha$-reductase activity in $B_3$ cells suggests that the enzyme is localized in Leydig cells, because at each age $B_3$ cells had a greater percentage of Leydig cells in the present study. Although $5\alpha$-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 $5\alpha$-reductase activity is localized exclusively in Leydig cells remains to be determined. Previous studies identified $5\alpha$-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 $5\alpha$-reductase activity is present in these cells.

The pattern of $17\beta$-HSD activity observed in both $B_2$ and $B_3$ cells in the present study confirms the maturation of Leydig cells. Although $5\alpha$-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 $5\alpha$-reductase activity is localized exclusively in Leydig cells remains to be determined. Previous studies identified $5\alpha$-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 $5\alpha$-reductase activity is present in these cells.

The pattern of $17\beta$-HSD activity observed in both $B_2$ and $B_3$ cells in the present study confirms the maturation of Leydig cells. The pattern of $17\beta$-HSD activity observed in both $B_2$ and $B_3$ cells in the present study confirms the maturation of Leydig cells.
3α-HSD activity is higher in B2 cells. The increase in 5α-ane-3α-HSD paralleled the maturational increase in 5α-reductase and 17β-HSD; however, after peaking between days 30–40, activity remained elevated. The only other maturational study estimating 5α-ane-3α-HSD in rat testes reported that activity gradually increased between days 10–60 when activity was expressed as nmol 3α-diol/pair testes (29). They used the soluble fraction of whole testes and included NADPH in their incubations. Because few Leydig cells localize in B2, this may suggest that 5α-ane-3α-HSD is concentrated in a cell type other than Leydig cells.

Acknowledgments

These studies were supported by the Veterans Administration. I would like to express appreciation to Ms Vicki Fisher-Simpson for excellent technical assistance, and to Mrs Ann Martin for secretarial assistance.

References

24. Milewich L, Chen GT, Lyons C, Tucker TF, Uhr JW, MacDonald DC. Metabolism of androstenedione by


Received February 21st, 1989.
Accepted May 30th, 1989.

Dr Eisuke P. Murono,
Department of Medicine,
University of South Carolina School of Medicine,
Administration Building,
Columbia, SC 29208, USA.