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Variation in the End Products of Androgen Biosynthesis and Metabolism during Postnatal Differentiation of Rat Leydig Cells*

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ABSTRACT

The amount of testosterone (T) secreted by Leydig cells is determined by a balance between T biosynthetic and metabolizing enzyme activities. It has been established that 5α-androstan-3α,17β-diol (3α-DIOL) is the predominant androgen secreted by the testes of immature rats during days 20–40 postpartum, whereas T is the major androgen by day 56. However, the underlying changes in T biosynthetic and metabolizing enzymes during Leydig cell development and their magnitudes have remained unclear. The aim of the present study was to define the developmental trends for T biosynthetic and metabolizing enzymes in Leydig cells at three distinct stages of pubertal differentiation: mesenchymal-like progenitors on day 21, immature Leydig cells on day 35, and adult Leydig cells on day 90. Production rates for precursor androgen (androstenedione), T, and 5α-reduced androgens [androsterone (AO) and 3α-DIOL] were measured in progenitor, immature, and adult Leydig cells in spent medium after 3 h *in vitro*. Steady state messenger RNA (mRNA) levels and enzyme activities of biosynthetic and metabolizing enzymes were measured in fractions of freshly isolated cells at each of the three stages. Unexpectedly, progenitor cells produced significant amounts of androgen, with basal levels of total androgens (androstenedione, AO, T, and 3α-DIOL) 14 times higher than those of T alone. However, compared with immature and adult Leydig cells, the capacity for steroidogenesis was lower in progenitor cells, with a LH-stimulated production rate for total androgens of 84.33 ± 8.74 ng/10^6 cells·3 h (mean ± SE) *vs.* 330.13 ± 44.19 in immature Leydig cells and 523.23 ± 67.29 in adult Leydig cells. The predominant androgen produced by progenitor, immature, and adult Leydig cells differed, with AO being released by progenitor cells (72.08 ± 9.02% of total androgens), 3α-DIOL by immature Leydig cells (73.33 ± 14.52%), and T by adult Leydig cells (74.38 ± 14.73%). Further examination indicated that changes in the predominant androgen resulted from differential gene expression of T biosynthetic and metabolizing enzymes. Low levels of type III 17β-hydroxysteroid dehydrogenase (17βHSD) mRNA and enzyme activity were present in progenitor cells compared with immature and adult Leydig cells. In contrast, levels of type I 5α-reductase (5αR) and 3α-hydroxysteroid dehydrogenase (3αHSD) mRNA and enzyme activities were dramatically lower in adult Leydig cells compared with those in progenitor and immature Leydig cells. Several T biosynthetic enzymes attained equivalent levels in immature and adult Leydig cells, but T was rapidly metabolized in the former to 3α-DIOL by high 5αR and 3αHSD activities, which were greatly reduced in the latter. Therefore, declines in 5αR and 3αHSD activities are hypothesized to be a major cause of the ascendancy of T as the predominant androgen end product produced by adult Leydig cells. These results indicate that steroidogenic enzyme gene expression is not induced simultaneously, but through sequential changes in T biosynthetic and metabolizing enzyme activities, resulting in different androgen end products being secreted by Leydig cells during pubertal development. (*Endocrinology* **139:** 3787–3795, 1998)

ANDROGEN stimulation is responsible for the maintenance of spermatogenesis and secondary sexual characteristics in the male. According to the literature, testosterone (T) produced by Leydig cells of the testis is the major androgen in the circulation of men and adult males of most mammalian species, including the rat. However, in rats, which have been studied more extensively than most other species, 5α-androstan-3α,17β-diol (3α-DIOL) and androsterone (AO) are abundant in the circulation between days 20–40 postpartum when T is still low (1). Testicular tissue of rats aged 15–40 days metabolizes radiolabeled progesterone and T to 3α-DIOL and, to a lesser extent, AO (2–5). Testicular enzyme activities of 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase/C17–20 lyase ($P450_{c17}$), and 17β-hydroxysteroid dehydrogenase (17βHSD) increase gradually between the ages of 20 and 60 days and plateau thereafter (6, 7). The amount of testicular 5α-reductase (5αR) activity, on the other hand, sharply increases between days 20 and 40, and then falls between days 40 and 60 (7). Further analysis demonstrated that type I 5αR and 3α-hydroxysteroid dehydrogenase (3αHSD) messenger RNA (mRNA) and protein are abundantly present in progenitor and immature Leydig cells during days 15–35 (8, 9). These findings suggest that the Leydig cell itself is a metabolizing site for androgens during puberty, at least via the 5α-reduction pathway. However, technical barriers prevented the testing of this hypothesis, because steroidogenic enzymes such as 17βHSD (10–12), 5αR (13, 14), and 3αHSD (14, 15) are present in other cell types in the testis. Therefore, purified Leydig cells were examined in the present study to delineate T biosynthesis and metabolism occurring in this cell type during pubertal differentiation.

Androstenedione (DIONE; a precursor of T), T, and the 5α-reduced metabolites, AO and 3α-DIOL, were measured in purified progenitor Leydig cells on day 21, in immature Leydig cells on day 35, and in adult Leydig cells on day 90. The predominant androgen end product varied depending on the stage of differentiation; it was AO in progenitor Leydig cells, 3α-DIOL in immature Leydig cells, and T in adult Leydig cells. These results led to a further examination of T biosynthetic [cholesterol side-chain cleavage enzyme

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($P450_{scc}$), 3βHSD, $P450_{c17}$, and 17βHSD] and metabolizing (5αR and 3αHSD) enzyme activities in Leydig cells. The results showed that activities of T biosynthetic and metabolizing enzymes in developing Leydig cells determine not only their capacity for T production but also the predominant androgen end product that is secreted.

Materials and Methods

Chemicals

25-[26,27-^{3}H]Hydroxycholesterol, [7-N-^{3}H]pregnenolone, [1,2-N-^{3}H]17α-hydroxyprogesterone, [1β,2β-N-^{3}H]androst-4-ene-3,17-dione, [1,2,6,7-N-^{3}H]T, [1,2-N-^{3}H]dihydrotestosterone, 5α-[9,11-N-^{3}H]androstane-3α,17β-diol, and [9,11-N-^{3}H]AO were purchased from DuPont-New England Nuclear (Boston, MA). [1,2,6,7-N-^{3}H]Progesterone was purchased from Amersham International (Aylesbury, UK). Nonradioactive steroids were purchased from Sigma Chemical Co. (St. Louis, MO) or Steraloids (Wilton, NH). 4-Methyl-aza-3-oxo-5α-pregnan-20(*S*)-carboxylate, an inhibitor of 5αR, was provided by Merck (Rahway, NJ). The antibodies for 3α-DIOL and AO in RIA were provided by Dr. D. T. Armstrong (Department of Obstetrics and Gynecology, University of Western Ontario, London, Ontario, Canada).

Animals

Sprague-Dawley rats (dams with litters of male pups, immature males, and adult males) were purchased from Charles River Laboratories (Wilmington, MA). The males rats were 21, 35, and 90 days of age on the day of Leydig cell isolation. The animals were killed by asphyxiation with CO_2. The animal protocol was approved by the institutional animal care and use committee of the Rockefeller University (Protocol 91200). A complete description of the procedure that was used to isolate each of the three stages of Leydig cell differentiation has been published (16, 17). The purity of cell fractions was evaluated by histochemical staining for 3βHSD activity with 0.4 mM etiocholanolone as the steroid substrate (18). Enrichment of the three fractions was typically more than 95%. The absence of androgen-binding protein mRNA in the cell fractions demonstrated that there was no appreciable contamination by Sertoli cells.

Androgen production

Isolated progenitor Leydig cells, immature Leydig cells, and adult Leydig cells were incubated at a concentration of $0.1–0.25 \times 10^6$ cells/ml in Leydig cell culture medium consisting of DMEM and Ham's F-12 medium (D2906, Sigma Chemical Co.) buffered with 15 mM HEPES and 14 mM $NaHCO_3$ and containing 1% BSA for 3 h at 34 C in a shaking water bath. Incubations of triplicate samples were conducted in medium alone (basal) or in medium plus a maximally stimulating dose of ovine LH (100 ng/ml). At the end of 3 h, the samples were centrifuged at $500 \times g$. Supernatants were extracted with 2 ml ethyl acetate twice, and the organic layer was dried under nitrogen gas. Steroids in the samples were fractionated using Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column chromatography as previously described (19). The elution system was chloroform-butane-ethanol (50:50:1, vol/vol/vol) saturated with distilled water. Clear separation of DIONE, AO, T, and 3α-DIOL in this system was confirmed using radiolabeled steroids (19). The recovery rates following extraction and column separation (DIONE, 90.7 ± 5.8%; AO, 76.2 ± 2.0%; T, 73.9 ± 3.7%; 3α-DIOL, 86.0 ± 1.2%) were used to correct the final concentration measured by RIA. RIAs of DIONE, AO, T, and 3α-DIOL were performed as previously described (20–22). The results of three separate experiments were averaged for statistical analysis.

Enzyme assay

With the exception of $P450_{scc}$, steroidogenic enzyme activities were measured by incubation of purified Leydig cells with radiolabeled substrates and separation of products by TLC as previously described (23–25). The substrate concentrations used for each enzyme were maximal to ensure that the concentration of substrate was not rate limiting. Control samples of culture medium alone were run in parallel with each enzyme assay. Briefly, reaction mixture (0.5 ml) was prepared in Leydig cell medium that contained 1 μM substrate (1 μCi) in medium. As T undergoes 5α-reduction in immature Leydig cells, 4-methyl-aza-3-oxo-5α-pregnan-20(*S*)-carboxylate (2 μM) was used to inhibit 5αR when $P450_{c17}$ or 17βHSD was measured. The reaction mixture was maintained at pH 7.2. Reactions were initiated by adding to the reaction medium an aliquot of $0.1–0.2 \times 10^6$ Leydig cells. The reaction mixtures, conducted in triplicate, were maintained at 34 C in a shaking water bath for 10 min. Reactions were terminated by adding ice-cold ethyl acetate, and steroids were rapidly extracted. The organic layer was dried under nitrogen. The radioactivity was measured using a radiometric scanner (System 200/AC3000, Bioscan, Washington DC). The activity of 3βHSD was determined by measuring conversion of pregnenolone to progesterone. $P450_{c17}$ catalyzes two mixed function oxidase reactions: 17α-hydroxylation and C17–20 cleavage. The activity of 17α-hydroxylation was determined by measuring conversion of progesterone to 17α-hydroxyprogesterone, DIONE, and T. The activity of C17–20 cleavage was determined by measuring conversion of 17α-hydroxyprogesterone to DIONE and T. The activity of 17βHSD was determined by measuring the conversion of DIONE to T. The activity of 5αR was determined by measuring the conversion of T to dihydrotestosterone, 3α-DIOL, and 5α-androstane-3β,17β-diol. The activity of 3αHSD was determined by measuring the conversion of dihydrotestosterone to 3α-DIOL. The steroids were separated on TLC plates in chloroform-methanol (97:3) for 3βHSD, 17βHSD, and 5αR assays; chloroform-ether (7:1, vol/vol) for $P450_{c17}$ 17α-hydroxylation and C17–20 cleavage assays; and diethyl ether-acetone (98:2) for the 3αHSD assay.

Activity of $P450_{scc}$ was determined by measuring the conversion of side-chain labeled 25-[26,27-^{3}H]hydroxycholesterol to radioactive 4-hydroxyl-4-methyl-pentanoic acid as previously described (26). Leydig cells were incubated in a total volume of 0.5 ml medium containing 1 μCi 25-[26,27-^{3}H]hydroxycholesterol (1 μM 25-hydroxycholesterol). Incubations were performed for 30 min at 34 C, and at the end of the incubation 0.5 ml NaOH (0.5 M) was added. The mixture was extracted twice with 2 ml chloroform and mixed with neutral alumna to remove nonmetabolized substrate (26), and an aliquot was removed for measurement by liquid scintillation counting.

RT-PCR

Within the cytochrome P450 enzyme superfamily, $P450_{scc}$ is encoded by a single $P450_{scc}$ gene (designated CYP11A1), and $P450_{c17}$ is encoded by a single $P450_{c17}$ gene (designated CYP17). However, the existence of multiple isoforms of hydroxysteroid dehydrogenases and 5αR have been demonstrated. In the rat, four distinct 3βHSD complementary DNAs (cDNAs) have been identified (27). These four isoforms share 76–94% identity in their amino acid sequences (28, 29). Types I and II are the only isoforms that are expressed in rat testis (28).

Four distinct 17βHSD cDNAs have been cloned in several species (reviewed in Ref. 30). Although three isoforms have been identified in the rat (30), it is believed that the type III 17βHSD isoform is the major protein in the testis responsible for the conversion of DIONE to T (31). In the rat, however, type III 17βHSD cDNA has not been cloned. Therefore, we designed a pair of primers to amplify the common sequence of human and mouse type III 17βHSD (31, 32). The primers amplified a 360-bp product from Leydig cell cDNA. The PCR product was partially sequenced and analyzed with respect to the known isoforms (rat types I, II, and IV 17βHSD, type III human and mouse 17βHSD) (31–35) using the LFASTA program (36).

At least three isoforms of 3αHSD have been cloned in several species (37), and the type I isoform has been cloned from rat liver cDNA (38–40). This isoform has been demonstrated to be present in Leydig cells (7). The two isoforms of 5αR that have been cloned in the rat are present in rat testis (41, 42).

Therefore, using the published sequences of the rat $P450_{scc}$ (43), types I and II 3βHSD (28), $P450_{c17}$ (44), types I and II 5αR (41, 42), type I 3αHSD (39), and ribosomal protein S16 (RPS16) (45), primers were selected using PRIMER software (Whitehead Institute of Biomedical Research, Cambridge, MA) and synthesized on an oligonucleotide synthesizer (Gene Assembler Special, LKB, Rockville, MD). Table 1 summarizes the properties of the primers.

Total RNA was extracted from isolated Leydig cells by a single step

TABLE 1. Parameters of oligonucleotide primer pairs for seven target genes

Target mRNA (Ref.)	Localization and sequence of pair	GenBank or EMBL	Size of PCR product (bp)	Identification
P450scc (43)				
Forward	5′-AGGTGTAGCTCAGGACTTCA-3′	gb (J05156)[a]	399	RE (*Fok* I)[b]
Reverse	5′-AGGAGGCTATAAAGGACACC-3′			
3β-HSD (I, II) (28)				
Forward	5′-TCACATGTCCTACCCAGG-3′	gb (M38178)	264	RE (*Taq* I)[b]
Reverse	5′-ATTTTTCAGGATGCTCCC-3′	gb (M38179)		
P450c17 (44)				
Forward	5′-TCATCAAGAAGGGAAAAGAA-3′	gb (M31681)	294	RE (*Fok* I)[b]
Reverse	5′-TGAAGCAGATAGCACAGATG-3′			
17β-HSD (III) (32)				
Forward	5′-TCAATGGGACAATGGGCAGT-3′	gb (U66827)	363	Sequencing
Reverse	5′-GCYGYGYCAYCYYGACYACG-3′	gb (U05659)		
5α-R (I) (41)				
Forward	5′-CAATCCTGCAAGATTCCACC-3′	gb (J05035)	380	RE (*Eco*R I)[b]
Reverse	5′-ATTGGTCCTTGGGTGCATTC-3′			
5α-R (II) (42)				
Forward	5′-ACCACCACACAGGTTCTACCTT-3′	gb (M95058)	519	RE (*Eco*R I)[b]
Reverse	5′-CTGAGTCAGGATTAGGTTGC-3′			
3α-HSD (39)				
Forward	5′-CCCATCGATGCGTAACAAG-3′	gb (M61937)	237	RE (*Fok* I)[b]
Reverse	5′-GCCGTGCTTCAATCTTGCTT-3′			
RPS16 (45)				
Forward	5′-AAGTCTTCGGACGCAAGAAA-3′	emb (X17665)	148	RE (*Pst* I)[b]
Reverse	5′-GACAAGACGAAGACCCGTT-3′			

[a] The sequence accession number from GenBank (gb) or EMBL (emb).
[b] The restriction endonuclease (RE) was listed.

method, using phenol and guanidinium thiocyanate (Trireagent, Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Leydig cell total RNA (400 ng) was reverse transcribed with avian myeloblastosis virus reverse transcriptase according to a previously described procedure for RT-PCR (46). Briefly, total RNA samples were reverse transcribed using random hexamers plus deoxyNTPs at 42 C for 75 min, and the reaction was terminated by heating at 95 C for 5 min. Target cDNA was coamplified with RPS16 cDNA, an internal standard. PCR was initiated by addition of *Taq* DNA polymerase in the presence of [α-^{32}P]deoxy-CTP, and proceeded for 30 cycles at an annealing temperature of 52 C. Radiolabeled PCR bands were visualized by exposure to autoradiographic film. Quantitative analysis of mRNA levels was performed by scanning the films in a laser densitometer (Ultroscan, LKB). The signal intensities for steroidogenic enzyme mRNAs were normalized to RPS16. Preliminary studies showed that the targeted cDNAs were amplified linearly between 15–35 cycles of PCR.

Statistics

All measurements were repeated at least three times. The data were analyzed by the Kruskal-Wallis ANOVA, followed by multiple comparisons testing to identify significant differences between groups (47).

Results

Androgens produced by Leydig cells

Stage-dependent differences were observed in Leydig cell capacity for T production. The basal T production was, respectively, 4 and 30 times higher in immature and adult Leydig cells compared with that in progenitor Leydig cells. When the total androgen profile was measured, however, the stage-dependent differences were less pronounced (Table 2). The three Leydig cell preparations showed, respectively, 2-, 6-, and 6-fold increases in total androgen production in response to LH stimulation compared with basal control values (Table 2). These results indicated that progenitor Leydig cells were less responsive to LH stimulation than immature and adult Leydig cells.

Further analysis of the total androgen profile showed that the primary androgen end products produced by progenitor, immature, and adult Leydig cells were different. AO was the primary androgen secreted by progenitor Leydig cells because this steroid constituted 72.08 ± 9.02% of the total androgens released under LH-stimulated conditions. Parallel measurements showed that 3α-DIOL was the primary androgen for immature Leydig cells (73.33 ± 14.52% of total androgens), and T was primary androgen for adult Leydig cells (74.38 ± 14.73%; Fig. 1).

Synthesis of T and 3α-DIOL requires 17βHSD activity, and the low release rates for these steroids from progenitor cells suggest that the amount of 17βHSD is low in these cells. AO and 3α-DIOL require the activities of 5αR and 3αHSD, and the high release rates of these steroids by progenitors and immature Leydig cells indicate that androgen-metabolizing enzymes are most highly expressed in Leydig cells before the completion of puberty and then decline in adulthood.

Steroidogenic enzyme activities

The underlying basis for the different profiles of androgen release from three distinct stages of Leydig cell differentiation was examined further by measuring T biosynthetic and metabolizing enzyme activities in purified cells. Steroidogenic enzyme activities were measured in intact Leydig cells, because homogenization can change the relative rates of oxidative and reductive activities in most hydroxysteroid dehydrogenases. The first step of T biosynthesis is the con-

TABLE 2. Leydig cell capacity for androgen production during postnatal development

Conditions	Age group	Total androgen (ng/10^6 cells · 3 h)	Ratio of 17-hydroxyl to 17-keto androgen	Ratio of 5α-reduced to 5α-oxidized androgen
Basal	Progenitor Leydig cells	36.77 ± 5.32^a	0.17 ± 0.05^a	8.45 ± 1.49^a
	Immature Leydig cells	55.10 ± 10.41^a	4.97 ± 0.76^b	2.69 ± 0.57^a
	Adult Leydig cells	89.33 ± 4.13^b	5.71 ± 0.87^b	0.12 ± 0.02^b
+LH	Progenitor Leydig cells	84.33 ± 8.74^b	0.20 ± 0.03^a	4.92 ± 0.08^a
	Immature Leydig cells	330.13 ± 44.19^c	4.88 ± 1.33^b	5.41 ± 0.94^a
	Adult Leydig cells	523.23 ± 69.29^c	4.55 ± 1.33^b	0.09 ± 0.02^b

The incubations were performed in DMEM/F-12 alone (basal) or in medium plus ovine LH (+LH).
All data were expressed as the mean ± SE from four separate experiments.
$^{a-c}$ Groups in the same column sharing the same letter were not significantly different at $P < 0.05$.

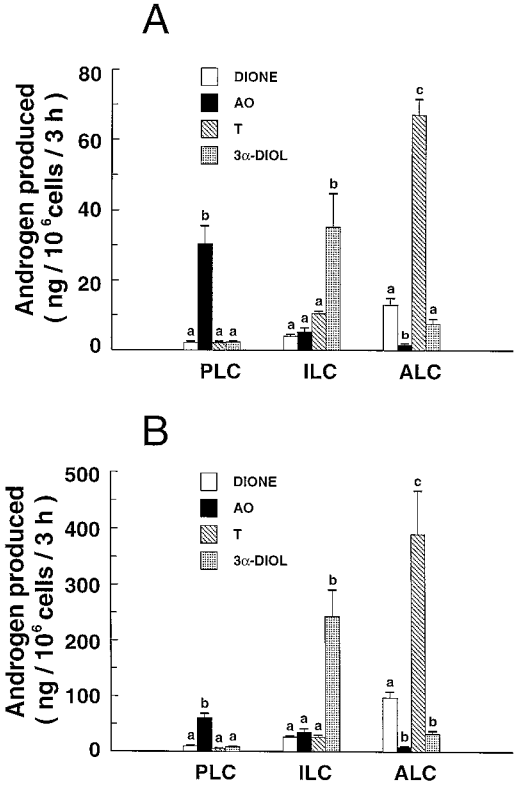

FIG. 1. Androgens produced by Leydig cells during pubertal development. Progenitor Leydig cells (PLC), immature Leydig cells (ILC), and adult Leydig cells (ALC) were isolated from 21-, 35-, and 90-day-old rats, respectively. The incubations were performed in DMEM-Ham's F-12 medium alone (basal; A) or in medium plus 100 ng/ml ovine LH (+ LH; B) with 0.1–0.25 × 10^6 cells. Measurement of DIONE, AO, T, and DIOL was performed as described in *Materials and Methods*. The levels of different androgens were expressed as the mean ± SE in duplicate samples from four sets of separate experiments. Groups sharing the same alphabet letter were not significantly different at $P < 0.05$.

version of cholesterol to pregnenolone, which is catalyzed by $P450_{scc}$. The conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids is catalyzed by 3βHSD. $P450_{c17}$ catalyzes both the 17α-hydroxylase and C17–20 lyase reactions to produce DIONE (48). The final step of T biosynthesis is catalyzed by 17βHSD reductive activity. DIONE and T can be metabolized by 5αR and 3αHSD into AO and 3α-DIOL, respectively. As shown in Fig. 2, the level of $P450_{scc}$ was low in progenitor Leydig cells and increased during Leydig cell differentiation. 3βHSD and $P450_{c17}$ (17α-hydroxylation and C17–20 cleavage) attained half of their mature activity levels in progenitor cells, and levels in immature and adult Leydig cells were statistically equivalent. Progenitor cells had negligible 17βHSD reductive activity, which greatly increased in immature and adult Leydig cells.

There was a significant increase in 5αR activity during the transition from progenitor to immature Leydig cell, followed by a sharp decline to almost undetectable levels in adult Leydig cells. The levels of 3αHSD activity were highest in progenitor Leydig cells and lowest in adult Leydig cells (Fig. 2). These results indicate that changes in the activities of both T biosynthetic and metabolizing enzymes catalyze the formation of distinct androgen end products during Leydig cell development.

Steady state mRNA levels of steroidogenic enzymes

Differential changes in T biosynthetic and metabolizing enzyme activities could result from variation in the steady state levels of mRNAs that encode these enzymes. Therefore, the steady state mRNA levels of Leydig cell steroidogenic enzymes were evaluated. With the exception of $P450_{scc}$ and $P450_{c17}$, multiple isoforms of hydroxysteroid dehydrogenases and 5αR have been identified. Four distinct 3βHSD cDNAs have been found in the rat (27), and rat Leydig cells are known to express 3βHSD isoforms I and II (28). Five distinct isoforms of 17βHSD have been identified in several species. Type III 17βHSD is thought to be responsible for catalyzing the conversion of DIONE to T in Leydig cells (31). Although it has not been cloned in the rat, type III 17βHSD is the predicted isoform for rat Leydig cells. A pair of primers was designed to identify sequences common to human and mouse type III 17βHSD cDNAs (31, 32). A 0.36-kb PCR product was identified in immature Leydig cells and adult Leydig cells (Fig. 3). Partial sequence analysis of the first 200 bp of the 0.36-kb PCR product showed that it had a 75% sequence similarity with mouse type III 17βHSD compared with only 35%, 40%, and 35% similarity with rat type I, II, and IV isoforms. Accordingly, this cDNA fragment was identified as type III. Two distinct isoforms of 5αR have been identified in the rat testis (9, 41). In the testis, type I is present exclusively in Leydig cells (9). Type I 5αR mRNA was abundant in progenitor and immature Leydig cells and almost undetectable in adult Leydig cells. No type II 5αR mRNA was detected in any stage of Leydig cell despite a clear signal in the positive control, the prostate (data not shown). Of the three isoforms of 3αHSD that have been identified, type I is known to be expressed in the testis (8). As shown in Fig. 3, when the

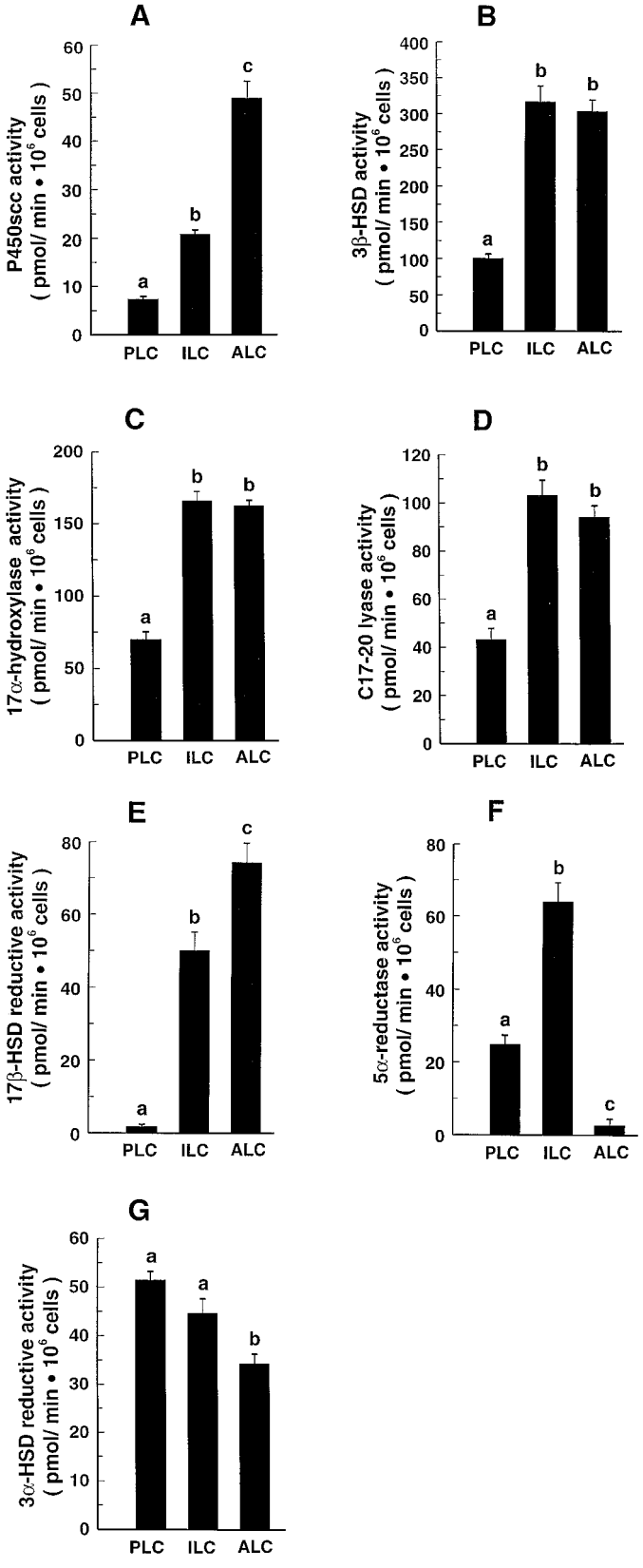

FIG. 2. Enzyme activities of T biosynthetic and metabolizing enzymes in intact Leydig cells during pubertal development. Progenitor Leydig cells (PLC), immature Leydig cells (ILC), and adult Leydig cells (ALC) were isolated from 21-, 35-, and 90-day-old rats, respectively. Determination of enzyme activity was performed as described in *Materials and Methods*. The enzyme activities of $P450_{scc}$ (A), 3βHSD (B), 17α-hydroxylase (C), C17–20 lyase (D), 17βHSD (E), 5α-reductase (F), and 3αHSD (G) were evaluated in PLC, ILC, and ALC. Values represent the mean ± SE from 9–15 different samples in 3–5 independent experiments. Shared alphabet letters denote groups that were not significantly different at $P < 0.05$.

steady state mRNA levels of these genes were evaluated, the trends for mRNAs corresponded closely with the trends defined for enzyme activities, suggesting that these genes transcriptionally regulate the levels of steroidogenic enzyme activity.

Discussion

From the data reported herein, it is apparent that development of the capacity for T production is achieved not only by increases in T biosynthetic enzymes but also by concurrent declines in T-metabolizing enzymes. Studies of serum and testicular androgen levels have shown that 5α-reduced androgens are high in immature males (1–5). In the rat, 3α-DIOL is the predominant androgen in circulation during days 20–40 (1). A distinction must therefore be drawn between steroidogenic ability and the capacity for T production. Steroidogenic ability is defined as the ability of cells to produce steroids, whereas the capacity for T production reflects the balance of both T biosynthetic and metabolizing enzyme activities. In the present study, using purified intact Leydig cells, we measured a 46-fold increase in basal T production during the transition from 21-day-old progenitor to 90-day-old adult Leydig cell under LH-stimulated conditions. In contrast, when the release rate was totaled for the four androgens, DIONE, T, AO, and 3α-DIOL, it only increased by 5-fold during the transition between progenitor and adult Leydig cell. This indicated that steroidogenic ability and capacity for T production are distinct measures of Leydig cell function. The steroidogenic ability of the progenitor Leydig cells, although lower than that of the more mature Leydig cell stages, is notable because these cells are devoid of smooth endoplasmic reticulum membranes that are the sites of steroidogenic enzyme localization. It is possible, therefore, that the steroidogenic ability of progenitor Leydig cells results from cytosolic, as opposed to membrane-bound, isoforms of steroidogenic enzymes. This is supported by the presence of type I 3αHSD, a cytosolic isoform, which attained its highest levels in progenitor Leydig cells. Another possible explanation is that mitochondrial isoforms of steroidogenic enzymes may be present in progenitor Leydig cells before the development of membrane-bound isoforms in the smooth endoplasmic reticulum. In this regard, a mitochondrial form of 3βHSD has been found to exist in bovine adrenal cortical cells (49). The primary androgen end product was different for each of the three stages of Leydig cell differentiation, with AO being the primary androgen released by progenitor Leydig cells, and 3α-DIOL and T, respectively, the primary androgens for immature and adult Leydig cells.

Synthesis of T and 3α-DIOL require 17βHSD reductive activity, and their low release rates from progenitor cells

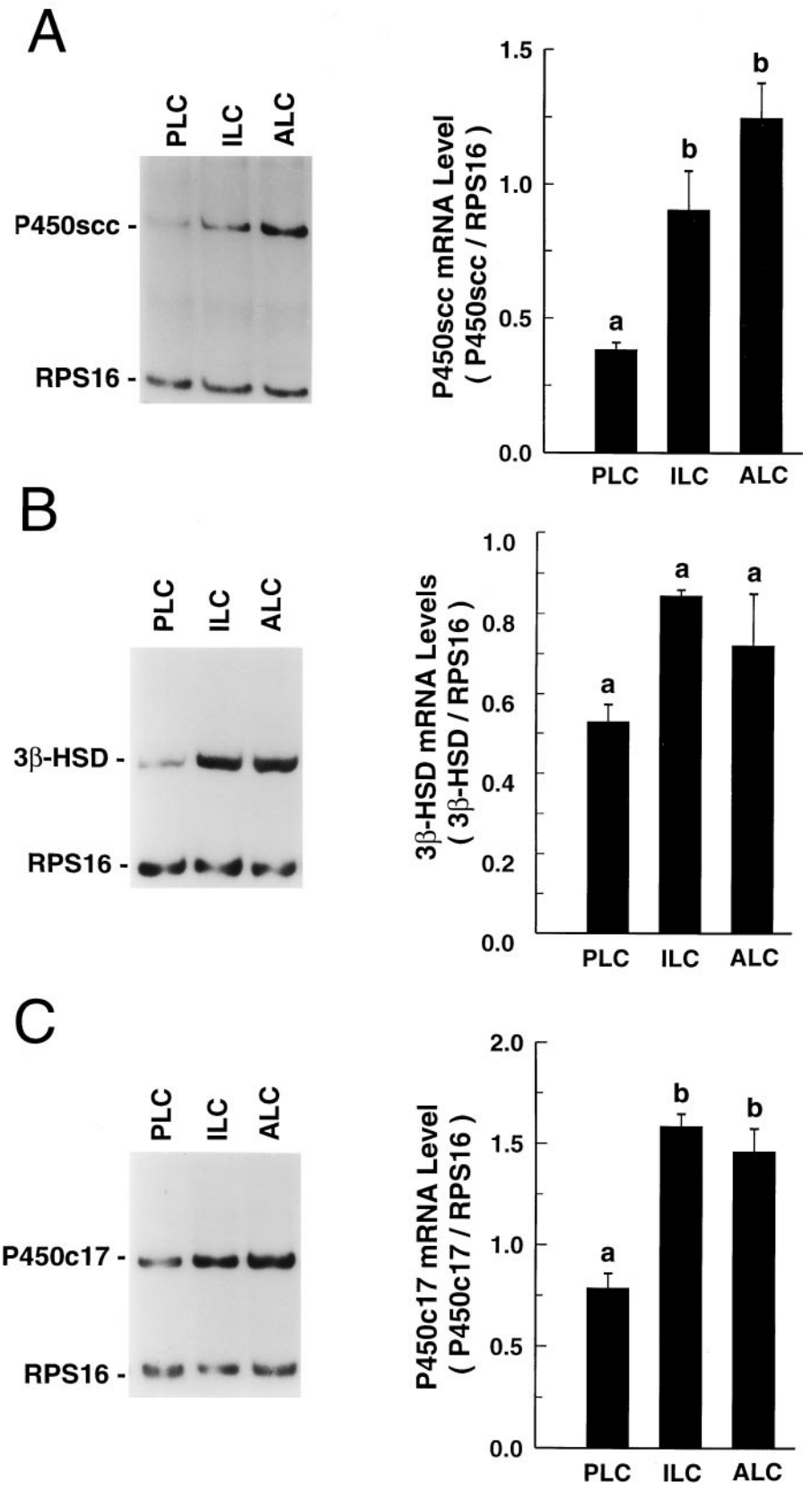

FIG. 3. Quantification of steady state mRNA levels for T biosynthetic and metabolizing enzymes in Leydig cells during pubertal development. Progenitor Leydig cells (PLC), immature Leydig cells (ILC), and adult Leydig cells (ALC) were isolated from 21-, 35-, and 90-day-old rats, respectively. Steady state mRNA levels of enzymes were measured by RT-PCR and normalized to RPS 16, an internal control. The steady state mRNA levels for $P450_{scc}$ (A), 3βHSD (B), $P450_{c17}$ (C), type III 17βHSD (D), type I 5α-reductase (E), and type I 3αHSD (F) were evaluated in PLC, ILC, and ALC. Values represent the mean ± SE from three different assays in three independent experiments. Shared alphabet letters denote groups were not significantly different at $P < 0.05$.

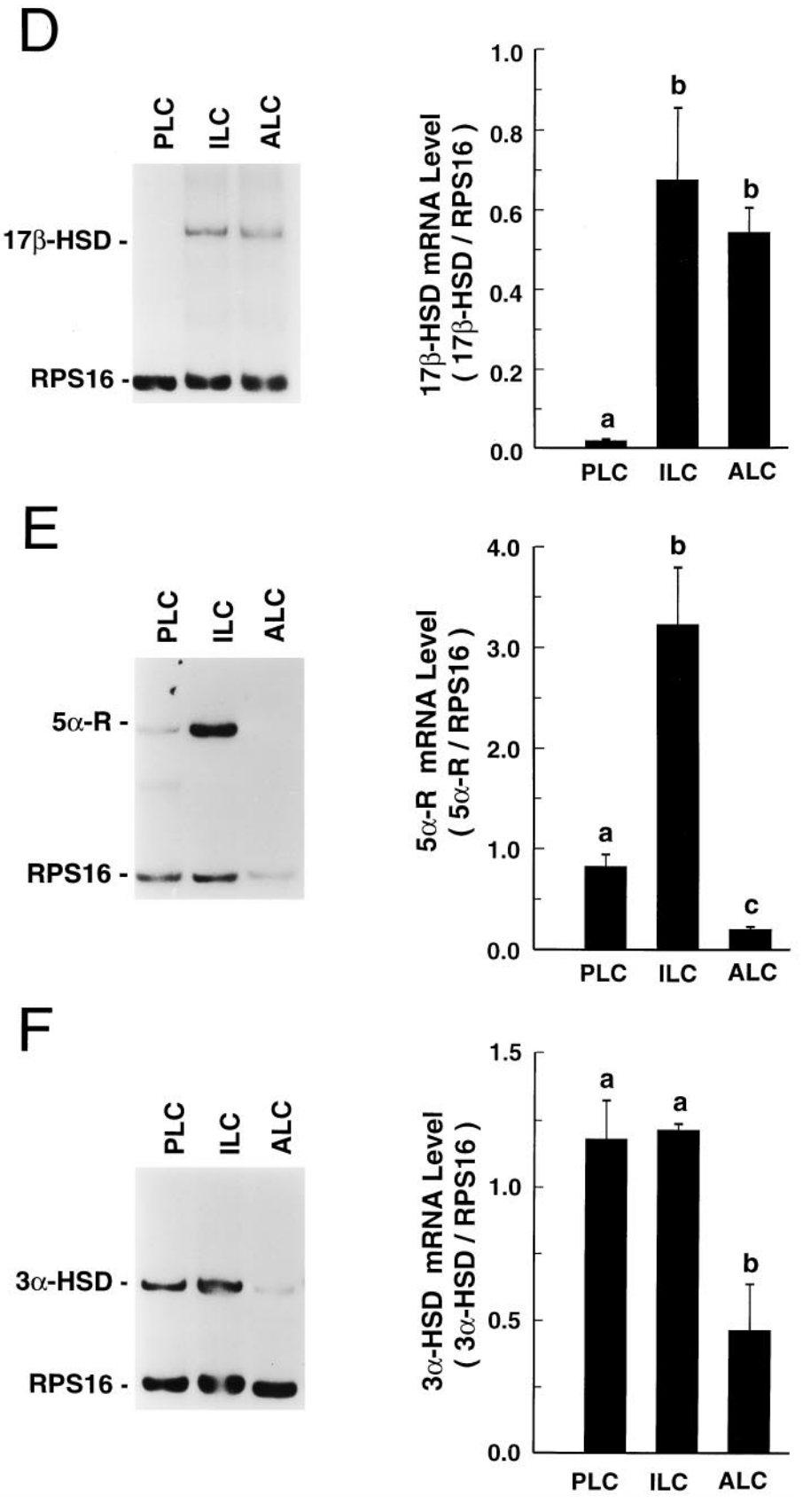

FIG. 3. Continued

suggest that 17βHSD activity is low in these cells. AO and 3α-DIOL require the activities of 5αR and 3αHSD, and the high release rates for these steroids by progenitors and immature Leydig cells indicate that androgen-metabolizing enzymes are abundantly expressed in Leydig cells before the completion of puberty, with declining levels in adulthood. Direct measurements of enzyme activities in purified cells explained the changes in end products released during Leydig cell differentiation. In progenitor cells, several androgen biosynthetic enzyme activities attained half of the more mature values, with the exception of 17βHSD, which was low, resulting in negligible conversion of DIONE to T. However, progenitor Leydig cells readily converted DIONE to 5α-androstanedione by 5αR and ultimately to the androgen end product, AO, through 3αHSD, because the activities of both 5αR and 3αHSD were high. Although T biosynthetic enzymes nearly all attained adult values in immature Leydig cells, T formed in these cells was rapidly metabolized to another androgen end product, 3α-DIOL. Adult Leydig cells contain significant amounts of 3αHSD activity. However, the absence of 5α-reductase in adult Leydig cells eliminates the catabolism of T, making this steroid the primary androgen end product, as T is not a substrate for 3αHSD. The 3αHSD activity present in adult Leydig cells may act to metabolize dihydrotestosterone, which is still produced at trace levels in the mature testis.

Steroidogenic enzyme activities have been measured in the developing testis (5–7). However, these studies report that 3α-DIOL is the primary androgen secreted from the immature testis on day 21 postpartum, when progenitor Leydig cells are present (2–5). Given the negligible 3α-DIOL production that was observed in progenitor Leydig cells, the predominance of this steroid in immature rat testis indicates that high levels of 17βHSD are expressed by other cell types in the testis. The seminiferous tubules and Sertoli cells in particular contain 17βHSD activity (10, 11). Indeed, the testis remains capable of 17βHSD activity when Leydig cells are completely destroyed by the Leydig cell toxicant ethylene-1,2-dimethanesulfonate, with only a 50% decline in conversion of DIONE to T (12).

The present data indicate that the development of steroidogenic enzyme activities results from sequential, rather than simultaneous, induction of steroidogenic enzyme gene transcription. The steady state mRNA levels of steroidogenic enzymes were measured in Leydig cells to evaluate the relationship between mRNA levels and enzyme activity. We demonstrated that mRNA levels for T biosynthetic enzymes were significantly lower in progenitor Leydig cells than in immature and adult Leydig cells. Of the T biosynthetic enzymes, type III 17βHSD was lowest in progenitor cells, consistent with the enzyme activity data. The presence of type III 17βHSD mRNA in rat Leydig cells indicated that this isoform encodes Leydig cell-specific 17βHSD, which is also expressed in human and mouse testes (31, 32). Levels of type I 5αR were highest in immature Leydig cells, followed by progenitor and adult Leydig cells, confirming the transitory expression of this enzyme activity during pubertal development. Although type II 5αR is reported to be in rat testis (8, 42), this isoform was undetectable in Leydig cells.

In summary, these data demonstrate that biosynthetic and androgen-metabolizing capacities are separately modulated in Leydig cells during pubertal differentiation and that this has significant consequences for the overall rate of T production. Progenitor Leydig cells primarily produce AO, with 3α-DIOL predominant in immature Leydig cells and T predominant in adult Leydig cells. During the transition from progenitor to immature Leydig cells, increased type III 17βHSD mRNA and enzyme activities confer adult levels of T biosynthetic capacity. During the later transition from immature to adult Leydig cell, loss of type I 5αR and decline of type I 3αHSD lower androgen metabolism, making T the primary androgen end product.

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References

1. **Moger WH** 1977 Serum 5α-androstane-3α,17β-diol, androsterone, and testosterone concentrations in the male rat. Influence of age and gonadotropin stimulation. Endocrinology 100:1027–1032
2. **Chubb C, Ewing LL** 1981 Steroid secretion by sexually immature rat and rabbit testis perfused *in vitro*. Endocrinology 109:1999–2003
3. **Corpechot C, Baulieu EE, Robel P** 1981 Testosterone, dihydrotestosterone and androstanediols in plasma, testes and prostates of rats during development. Acta Endocrinol (Copenh) 96:127–135
4. **Inano H, Tamaoki B-I** 1966 Bioconversion of steroids in immature rat testes *in vitro*. Endocrinology 79:579–590
5. **Matsumoto K, Yamada M** 1973 5α-Reduction of testosterone *in vitro* by rat seminiferous tubules and whole testis at different stages of development. Endocrinology 93:253–255
6. **Payne AH, Kelch RP, Murono EP, Kerlan JT** 1977 Hypothalamic, pituitary and testicular function during sexual maturation of the male rat. J Endocrinol 72:17–26
7. **Goldman AS, Klingele DA** 1974 Developmental defects of testicular morphology and steroidogenesis in the male pseudohermaphrodite and response to testosterone and dihydrotestosterone. Endocrinology 94:1–16
8. **Shan L-X, Phillips DM, Bardin CW, Hardy MP** 1993 Differential regulation of steroidogenic enzymes during differentiation optimizes testosterone production by adult rat Leydig cells. Endocrinology 133:2277–2283
9. **Viger RS, Robaire B** 1995 Steady state steroid 5α-reductase messenger ribonucleic acid levels and immunocytochemical localization of the type I protein in the rat testis during postnatal development. Endocrinology 136:5409–5415
10. **Murono EP, Payne AH** 1976 Distinct testicular 17-ketosteroid reductase, one in interstitial tissue and one in seminiferous tubules. Differential modulation by testosterone and metabolites of testosterone. Biochim Biophys Acta 450:89–100
11. **Welsh MJ, Wiebe JP** 1978 Sertoli cell capacity to metabolize C19 steroids: variation with age and the effect of follicle-stimulating hormone. Endocrinology 102:838–844
12. **O'Shaughnessy PJ, Murphy L** 1991 Steroidogenic enzyme activity in the rat testis following Leydig cell destruction by ethylene-1,2-dimethanesulphonate and during subsequent Leydig cell regeneration. J Endocrinol 131:451–457
13. **Rivarola MA, Podesta EJ** 1972 Metabolism of testosterone-^{14}C by seminiferous tubules of mature rats: formation of 5α-androstan-3α,17β-diol-^{14}C. Endocrinology 90:618–623
14. **Dorrington JH, Fritz IB** 1975 Cellular localization of 5α-reductase and 3α-hydroxysteroid dehydrogenase in the seminiferous tubule of the rat testis. Endocrinology 96:879–889
15. **Wiebe JP** 1982 Identification of a unique Sertoli cell steroid as 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3α-DHP). Steroids 39:259–278
16. **Hardy MP, Kelce WR, Klinefelter GR, Ewing LL** 1990 Differentiation of Leydig cell precursors *in vitro*: a role for androgen. Endocrinology 127:488–490
17. **Shan LX, Hardy MP** 1992 Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. Endocrinology 130:1107–1114
18. **Payne AH, Downing JR, Wong KL** 1980 Luteinizing hormone receptors and testosterone synthesis in two distinct population of Leydig cells. Endocrinology 106:1424–1429
19. **Murphy BEP** 1971 'Sephadex' column chromatography as an adjunct to competitive protein binding assays of steroids. Nature New Biol 232:21–24
20. **Zamecnik J, Barbe G, Moger WH, Armstrong DT** 1977 Radioimmunoassays

for androsterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. Steroids 30:679–689
21. **Cochran RC, Ewing LL, Niswender GD** 1981 Serum levels of follicle stimulating hormone, 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, and 17β-estradiol from male beagles with spontaneous or induced benign prostatic hyperplasia. Invest Urol 19:142–147
22. **Thorneycroft IH, Ribairo WO, Stone SC, Tillson SA** 1973 A radioimmunoassay of androstenedione. Steroids 21:111–122
23. **O'Shaughnessy PJ, Payne AH** 1982 Differential effects of single and repeated administration of gonadotrophins on T production and steroidogenic enzymes in Leydig cell populations. J Biol Chem 257:11503–11509
24. **Murono EP, Washburn AL, Goforth DP** 1994 Enhanced stimulation of 5α-reductase activity in cultured Leydig cell precursors by human chorionic gonadotropin. J Steroid Biochem Mol Biol 48:377–384
25. **Cohen PE, Hardy MP, Pollard JW** 1997 Colony-stimulating factor-1 plays a major role in the development of reproductive function in male mice. Mol Endocrinol 11:1636–1650
26. **Georgiou M, Perkins LM, Payne AH** 1987 Steroid synthesis-dependent, oxygen-mediated damage of mitochondrial and microsomal cytochrome P-450 enzymes in rat Leydig cell cultures. Endocrinology 121:1390–1399
27. **Payne AH, Clarke TR, Bain PA** 1995 The murine 3β-hydroxysteroid dehydrogenase multigene family: structure, function and tissue-specific expression. J Steroid Biochem Mol Biol 53:111–118
28. **Zhao HF, Labrie C, Simard J, de Launoit Y, Trudel C, Martel C, Rheaume E, Dupont E, Luu-The V, Pelletier G, Labrie F** 1991 Characterization of rat 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase cDNAs and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. J Biol Chem 266:583–593
29. **Simard J, Durocher F, Mebarki F, Turgeon C, Sanchez R, Labrie Y, Couet J, Trudel C, Rheaume E, Morel Y, Luu-The V, Labrie F** 1996 Molecular biology and genetics of the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene family. J Endocrinol [Suppl] 150:S189–S207
30. **Labrie F, Luu-The V, Lin SX, Labrie C, Simard J, Breton R, Belanger A** 1997 The key role of 17β-hydroxysteroid dehydrogenases in sex steroid biology. Steroids 62:148–58
31. **Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW, Andersson S** 1994 Male pseudohermaphroditism caused by mutations of testicular 17β-hydroxysteroid dehydrogenase-3. Nat Genet 7:34–39
32. **Sha JA, Dudley K, Rajapaksha WRAKJS, O'Shaughnessy PJ** 1997 Sequence of mouse 17β-hydroxysteroid dehydrogenase type 3 cDNA and tissue distribution of the type 1 and type 3 isoform mRNAs. J Steroid Biochem Mol Biol 60:19–24
33. **Ghersevich SA, Nokelainen P, Poutsnen M, Orava M, Autio-Harmainen H, Rajaniemi H, Vihko R** 1994 Rat 17β-hydroxysteroid dehydrogenase type 1: primary structure and regulation of enzyme expression in rat ovary by diethylstilbestrol and gonadotropins *in vivo*. Endocrinology 135:1477–1487
34. **Akinola LA, Poutanen M, Vihko R** 1996 Cloning of rat 17β-hydroxysteroid dehydrogenase type 2 and characterization of tissue distribution and catalytic activity of rat type 1 and type 2 enzymes. Endocrinology 137:1572–1579
35. **Corton JC, Bocos C, Moreno ES, Merritt A, Marsman DS, Sausen PJ, Cattley RC, Gustafsson J-X** 1996 Rat 17β-hydroxysteroid dehydrogenase type IV is a novel peroxisome proliferor-inducible gene. Mol Pharmacol 50:1157–1166
36. **Pearson WR, Lipman DJ** 1988 Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448
37. **Penning TM** 1997 Molecular endocrinology of hydroxysteroid dehydrogenases. Endocr Rev 18:281–305
38. **Stolz A, Rahimi-Kiani M, Ameis D, Chan E, Ronk M, Shively JE** 1991 Molecular structure of rat hepatic 3α-hydroxysteroid dehydrogenase: a member of the oxidoreductase gene family. J Biol Chem 266:15253–15257
39. **Pawlowski JE, Huizinga M, Penning TM** 1991 Cloning and sequencing of the cDNA for rat liver 3α-hydroxysteroid, dihydrodiol dehydrogenase. J Biol Chem 266:8820–8825
40. **Cheng KC, White PC, Qin KN** 1991 Molecular cloning and expression of rat liver 3α-hydroxysteroid dehydrogenase. Mol Endocrinol 5:823–828
41. **Andersson S, Bishop RW, Russell DW** 1989 Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. J Biol Chem 264:16249–16255
42. **Normington KD, Russell DW** 1992 Tissue distribution and kinetic characteristics of at steroid 5α-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem 267:19548–19554
43. **Oonk RB, Krasnow JS, Beattie WG, Richards JS** 1989 Cyclic AMP-dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea. cDNA and deduced amino acid sequence of rat P-450scc. J Biol Chem 264:21934–219428
44. **Fevold HR, Lorence MC, McCarthy JL, Trant JM, Kagimoto M, Waterman MR, Mason JI** 1989 Rat P450–17α from testis: characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both Δ-4- and Δ-5-steroid-17,20-lyase reactions. Mol Endocrinol 3:968–975
45. **Chan YL, Paz V, Olvera J, Wool IG** 1990 The primary structure of rat ribosomal protein S16. FEBS Lett 263:85–88
46. **Shan L-X, Hardy DO, Catterall JF, Hardy MP** 1994 Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen acceptors, and steroidogenic enzymes in rat Leydig cell progenitors *in vivo*. Endocrinology 136:1686–1693
47. **Sokal RR, Rohlf FJ** 1995 Biometry, ed 3. Freeman, New York
48. **Nakajin S, Shively JE, Yuan P-M, Hall PF** 1981 Microsomal cytochrome P-450 from neonatal pig testis; two enzymatic activities (17α-hydroxylase and C17,20-lyase) associated with one protein. Biochemistry 20:4037–4042
49. **Cherradi N, Defaye G, Chambaz EM** 1994 Dual subcellular localization of the 3β-hydroxysteroid dehydrogenase isomerase: characterization of the mitochondrial enzyme in the bovine adrenal cortex. J Steroid Biochem Mol Biol 46:773–779