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Enzyme assay for 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1 activity in rat testis

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Abstract

The relative abundance and physiological role of 5α-reductase (5αR) isoforms in rat testis, in particular 5α-reductase Type 2 (5αR2) are poorly understood. Investigation of 5αR2 activity using enzyme kinetic studies was hampered by the high concentrations of 5α-reductase Type 1 (5αR1) in rat testis. Therefore, an assay was developed which exploited the differences in pH optima of the two isoforms. The 5αR assays measured the conversion of ^{3}[H]-testosterone to 5α-reduced metabolites (dihydrotestosterone + 3α-Androstanediol) at pH 5.0 and 7.0. To compensate for the overlap of 5αR1 activity at pH 5.0, the amount of 5αR1 activity at pH 5.0 was determined by measuring recombinant rat 5αR1 expressed in COS-7 cells at pH 5.0 and 7.0. The amount of activity at pH 5.0 that was attributed to 5αR1 was determined to be $12.4 \pm 1.4\%$ (mean ± S.D., $n = 14$). The 5αR2 assay was validated by determining recombinant rat 5αR2 activity in the presence of recombinant rat 5αR1 activity in COS cells. A $99.3 \pm 14.7\%$ recovery of 5αR2 activity was obtained when comparing 5αR2 activity recovered versus activity added. 5αR1 and 5αR2 activities were then assayed in rat testis extracts from 30, 75 and 147 days. Both isoforms markedly declined (50–100-fold) over this age range, with 5αR1 as the predominant isoform. In conclusion, an enzymatic assay that detects 5αR2 activity in the presence of high concentrations of 5αR1 was developed and is applicable in the measurement of 5αR2 activity in rat testis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: 5α-Reductase; Testis; Androgen; Spermatogenesis

1. Introduction

The steroid enzyme 5α-reductase (5αR, E.C. 1.3.99.5) metabolizes testosterone to dihydrotestosterone (DHT) [1]. Two genes encoding 5αR have been identified in rats [2–4] and these isoforms are referred to as 5αR Type 1 (5αR1) and Type 2 (5αR2), based on the chronological order in which they were discovered.

The 5αR isoforms have molecular weights of 28–29 k and are 46% identical in sequence, share similar substrate preferences and have similar gene structures [3,5,6]. They differ however in their pH optima for maximal enzymatic activity (pH 7 for 5αR1 and pH 5 for 5αR2), affinity for steroid substrates (micromolar K_m for 5αR1 and nanomolar K_m for 5αR2), expression levels and sensitivity to certain 4-azasteroid inhibitors [3,5].

The metabolism of testosterone to DHT by 5αR plays an important role in androgen physiology because DHT is a more potent androgen than testosterone [7,8]. The role of DHT in normal spermatogenesis is unknown. However, it has been shown that DHT plays a critical role in spermatogenesis at low testicular testosterone levels during experimentally-induced spermatogenic inhibition in adult rats [9,10].

RNA blotting studies in rat tissues indicate that 5αR1 mRNA predominates in androgen-independent tissues such as the skin and liver, whereas 5αR2 mRNA is the predominant isoform in reproductive tissues [3,4]. Exceptions to this general rule are the prostate and seminal vesicles which express similar amounts of both 5αR isoforms [4].

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The type of 5αR isoforms expressed in rat testis is controversial [4,11]. Early enzyme activity studies showed a peak in 5αR1 activity in rat testis between days 30 and 35, which decreased to undetectable levels with age [12–15]. More recently two studies using mRNA methodologies have investigated 5αR isoform expression in rat testis. One study [4] using RNA blotting reported the presence of predominantly 5αR2 in the testis (day 49) while the other study showed that only 5αR1 is expressed from days 7 to 91 by Northern blot analysis and immunoblotting [11]. The cellular localization of 5αR1 activity within the testis is also controversial with some studies showing either 5αR1 activity predominantly in the interstitium [16–19], predominantly in the seminiferous tubules [13,20], or in the seminiferous tubules only [14,15,21,22].

Currently, there is no quantitative procedure for measuring 5αR2 enzyme activity in the presence of 5αR1. 5αR2 activity can be assessed by making a ratio of the activities at pH 5.0 and 7.0, however this is only a relative indication of 5αR2 levels. In tissues which express large amounts of 5αR1 and low levels of 5αR2, measurement of 5αR2 activity is difficult because of the overlap of 5αR1 at pH 5. The aim of this study was to measure 5αR1 and 5αR2 enzyme activity in the testicular rat extracts. To the best of our knowledge, 5αR2 enzyme activity has not been investigated in rat testis of any age. In order to measure 5αR2 activity at pH 5 in the presence of high testicular 5αR1 activity, a method was devised to compensate for the overlapping effects of 5αR1 activity at pH 5.

2. Materials and methods

2.1. Reagents

4-Androsten-17β-ol-3-one (testosterone), 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT) and 5α-androstane-3α, 17β-diol (3α-Adiol) were purchased from Sigma (St Louis, MO). [1α, 2α-^{3}H(*N*)]-testosterone (53.5 Ci/mmol), [9,11-^{3}H(*N*)]-3α-Adiol (49.8 Ci/mmol), [4-^{14}C]-testosterone (57.3 mCi/mmol), and [4-^{14}C]-DHT (58.3 mCi/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). [4-^{14}C]-3α-Adiol was prepared by enzymatic reduction of [4-^{14}C]-DHT using 32-day rat testis (10 000 × *g* supernatant) as a source of 3α-hydroxy steroid dehydrogenase activity. The [4-^{14}C]-3α-Adiol produced was isolated by thin layer chromatography (TLC; see below). [4-^{14}C]-3α-Adiol and [1α, 2α-^{3}3H(*N*)]-testosterone were purified by TLC before use. Analytical grade chloroform, toluene, ethanol and diethyl ether (Merck, Kilsyth, Vic., Australia) were used for TLC. The coenzyme β-NADPH was obtained from Sigma (St Louis, MO).

2.2. Animals

Male Sprague–Dawley rats (30, 75 and 147 days) were obtained from the Monash Central Animal House and housed under 12-h light/dark cycle with free access to food and water. The study was approved by the Monash Medical Centre Animal Ethics Committee.

2.3. Tissue preparation

Animals were sacrificed by CO_2 asphyxiation. Testes, epididymis and liver were quickly excised, trimmed of fat, weighed and snap frozen in liquid nitrogen. Tissues were stored at −70°C until required for the 5αR activity assays.

Tissues were stored frozen until homogenization. Epididymis and liver were minced with a razor blade and homogenized (Heidolph Diax 600 homogenizer, Janke & Kunkel, Germany) in 0.25 M sucrose and stored at −70°C. Testes were decapsulated, homogenized in 0.25 M sucrose, centrifuged at 10 000 × *g* and the supernatant stored at −70°C.

2.4. 5α-Reductase activity assay

5α-Reductase activity was determined by measuring the conversion of ^{3}[H]-testosterone to ^{3}[H]-DHT + ^{3}[H]-3α-Adiol, following the protocol of Murono & Payne [23] with several modifications.

5α-Reductase assays were carried out in duplicate in a total volume of 0.55 ml. Radioactive (^{3}H-testosterone; 0.5 μCi) and nonradioactive (9.5 μM) testosterone, 0.5 mM β-NADPH (Sigma) and tissue extract (100 μl) in assay buffer (0.1 M Tris–citrate, pH 5.0 or 7.0) were incubated in a shaking water bath at 37°C for 20 min (epididymis and liver) or 60 min (testis and COS-7 cells). Controls were stored on ice during the incubation period. The assay was terminated by the addition of 2 ml 0.1 M NaOH. The recovery of steroids was monitored by the addition of [4-^{14}C]-testosterone, [4-^{14}C]-DHT and [4-^{14}C]-3α-Adiol. Nonradioactive steroids were also added prior to extraction to aid visualization on the chromatography plate. Samples were extracted with toluene:ether (1:5) and the aqueous phase frozen in an alcohol bath with dry ice. The organic phase containing the steroids was decanted, evaporated under N_2 and the residue dissolved in 10 μl ethanol.

The steroids (testosterone, DHT and 3α-Adiol) were separated by TLC on silica-impregnated glass fiber sheets (ITLC-SA, Gelman Sciences, Sydney, NSW, Australia). The eluting solvent system was chloroform:methanol (98:2, v/v) and the chromatographs were developed for 31 min. Testosterone was visualized by ultraviolet light at 254 nM and DHT and 3α-Adiol by iodine vapor. Visualization of 3α-Adiol was enhanced by spraying the plate with iodine vapor spot

enhancer (Alltech, Sydney, NSW, Australia). The chromatographic zones containing the steroids were cut out and placed into scintillation vials with Packard Emulsifier Safe scintillation fluid (Packard, Meridan, CA). The steroids were quantified by dual isotope liquid scintillation counting to measure both ^{3}H (conversion of testosterone to 5α-reduced metabolites) and ^{14}C (for estimation of steroid recovery) radioactivity.

5α-Reductase activity was expressed as the sum of the 5α-reduced metabolites (DHT + 3α-Adiol) formed from ^{3}H-testosterone after corrections for procedural losses. The recoveries of steroids as indicated by ^{14}C steroids were $47.2 \pm 5.1\%$ for testosterone, $47.3 \pm 5.0\%$ for DHT and $51.6 \pm 4.7\%$ for 3α-Adiol. Contaminating radioactivity in the DHT and 3α-Adiol region of the chromatogram was determined using control incubations, and were subtracted from DHT and 3α-Adiol values for the samples. The average background for the 5αR assays ($n = 6$) was 0.010 ± 0.003 pmol DHT + 3α-Adiol/min which corresponds to less than 0.12% conversion of testosterone.

Protein levels were determined by the BCA method (Pierce Chemical Co., Marrickville, NSW, Australia) using bovine serum albumin as standard.

2.5. Calculation of enzyme characteristics (K_m and V_{max})

The Michaelis–Menten constant (K_m) and V_{max} were determined by measuring 5α-reductase activity at pH 7.0 with 16 different concentrations of testosterone (1.9 nM–9.5 μM). The computer package Graphpad Prism (Graphpad Software, San Diego, CA) was used to analyze the enzyme kinetic data and Eadie–Scatchard plots [24] were used to graphically distinguish the presence of one or two isoforms.

2.6. Expression of recombinant rat 5α-reductase Type 1 and Type 2 in COS-7 cells

The cDNA clones encoding full length rat 5αR1 (pB5αRED1) and partial length 5αR2 (pT801) were kindly provided by Dr D.W. Russell and used as templates to amplify the coding region for subcloning into pcDNA 3.1$^{(+)}$ (Invitrogen, San Diego, CA) expression vectors.

Oligonucleotides synthesized by GIBCO (GIBCO Life Technologies, Melbourne, Vic., Australia) were designed with relevant restriction sites; *Eco*R1 and *Xho*1 for 5αR1, *Eco*R1 and *Xba*1 for 5αR2 (Boehringer and Mannheim, Melbourne, Vic., Australia). Forward and reverse primers for 5αR1 were [(5′-GCAGGAATTCAC CTC AGCTATGGAGTTGGATGAG-3′) and (5′-CAGACTCGAGTCACCAGGGA GACAGACAGAC-3′)] and forward and reverse primers for 5αR2 were [(5′-ACCAGAATTCACCACAG GCGAGATGCAGATTG-3′) and (5′-AGCATCTAGACAGTTCCTCCACA GAAACTTTGCTC-3′)]. Restriction sites are underlined.

Polymerase chain reaction (PCR) was used to amplify the coding region with 2.5 U Pfu DNA polymerase (Stratagene, Sydney, NSW, Australia), 1.25 mM dNTP, 50 pmol specific primers and 100 ng cDNA template. The amplification consisted of an initial denaturation step (94°C/2 min), 39 PCR cycles (annealing [55°C/2 min], extension [72°C/2 min], and denaturation [94°C/1 min]) and a final annealing (55°C/2 min) and extension step (72°C/7 min). The PCR products were cut with the appropriate restriction enzymes, purified and separated by electrophoresis on a 1.5% agarose gel and extracted using the QIAEX II Gel Extraction Kit (Qiagen, Clifton Hill, Vic., Australia).

The PCR product was subcloned into the pcDNA 3.1$^{(+)}$ expression vector using the Rapid DNA Ligation Kit (Boehringer and Mannheim) and transformed into DH5α competent cells (GIBCO). Recombinant plasmids were purified using the Concert High Purity Plasmid Purification System (GIBCO). Nucleotide sequencing of double-stranded plasmid DNA was performed with the Automated DNA Sequencer 377 (Perkin Elmer Biosystems, Scoresby, Vic., Australia) using the T7 priming site and the pcDNA 3.1$^{(+)}$/Bovine Growth Hormone reverse priming site (TAGAAGGCACAGTCGAGGC).

COS-7 cells (SV40-transformed monkey kidney cells) were plated at 5×10^{6} cells per cm^{2} in 175-cm^{2} culture flasks and grown in DMEM containing 10% (v/v) fetal bovine serum and supplemented with 7.5% sodium bicarbonate, 200 mM L-glutamine and 100 mM sodium pyruvate. The day before transfection, cells were plated in 100 mm culture dishes in serum-free medium to give ~50% confluency on the day of transfection. The transfection reagent Fugene-6 (Promega, Annandale, NSW, Australia) was used to transfect either vector alone (pcDNA 3.1$^{(+)}$) or recombinant rat 5αR1 or 5αR2 into COS-7 cells. The pAdvantage vector, containing the adenovirus VA1 gene was cotransfected with the individual cDNA's to enhance expression [25]. Transfection efficiency was measured by transfecting the pCMV-β-galactosidase plasmid. The media was replaced with serum-containing medium on days 2 and 3 and cells were harvested for 5αR activity assays on day 4. The cells were washed twice and frozen in 0.25 M sucrose. Cells were thawed and homogenized before use in the 5αR activity assay. The assay for recombinant 5α-reductases was carried out for 60 min at pH 7.0 (5αR1) and pH 5.0 (5αR2). All other conditions were as described above for the tissues.

2.7. Statistics

Differences between groups were assessed by independent t-tests at the level of $P < 0.05$. All data are expressed as mean ± S.D.

3. Results

3.1. Eadie–Scatchard plots to differentiate between 5α-reductase Type 1 and 2 enzyme activities

The 5αR isoforms can be differentiated at pH 7.0 based on their differences in affinity (K_m) for testosterone. The Eadie–Scatchard plots for male rat liver (Fig. 1A) and epididymis (Fig. 1B) showed the presence of two enzyme activities. The K_m value (Table 1) for 5αR1 in liver (2.41 μM) and epididymis (2.82 μM) is comparable to that obtained for recombinant rat 5αR1 (rec 5αR1, 1.64 μM).

Eadie–Scatchard plots from 30-day rat testis (Fig. 1E) showed the presence of both 5αR1 ($K_m = 2.25$ μM) and 5αR2 ($K_m = 0.13$ μM) activity, whereas 75-day rat testis (Fig. 1F) showed the presence of only 5αR1 ($K_m = 3.06$ μM). In rat testis at day 30, the V_{max} value for 5αR1 is 15-fold that of 5αR2 (Table 1), indicating that 5αR1 is the predominant isoform in rat testis. However, the V_{max}/K_m ratio for the two enzymes suggests that the conversion of testosterone to 5α-reduced metabolites would be attributed equally to both isoforms (1.66 and 1.98 for 5αR1 and 5αR2, respectively; Table 1).

3.2. pH profiles for 5α-reductase isoforms in rat testis

In order to develop an enzymatic assay for measuring 5αR2 activity in the presence of high concentrations of 5αR1, as is the case in rat testis, studies were undertaken to assess if differences in activities at their respective pH optima could be utilized to differentiate the two isoforms.

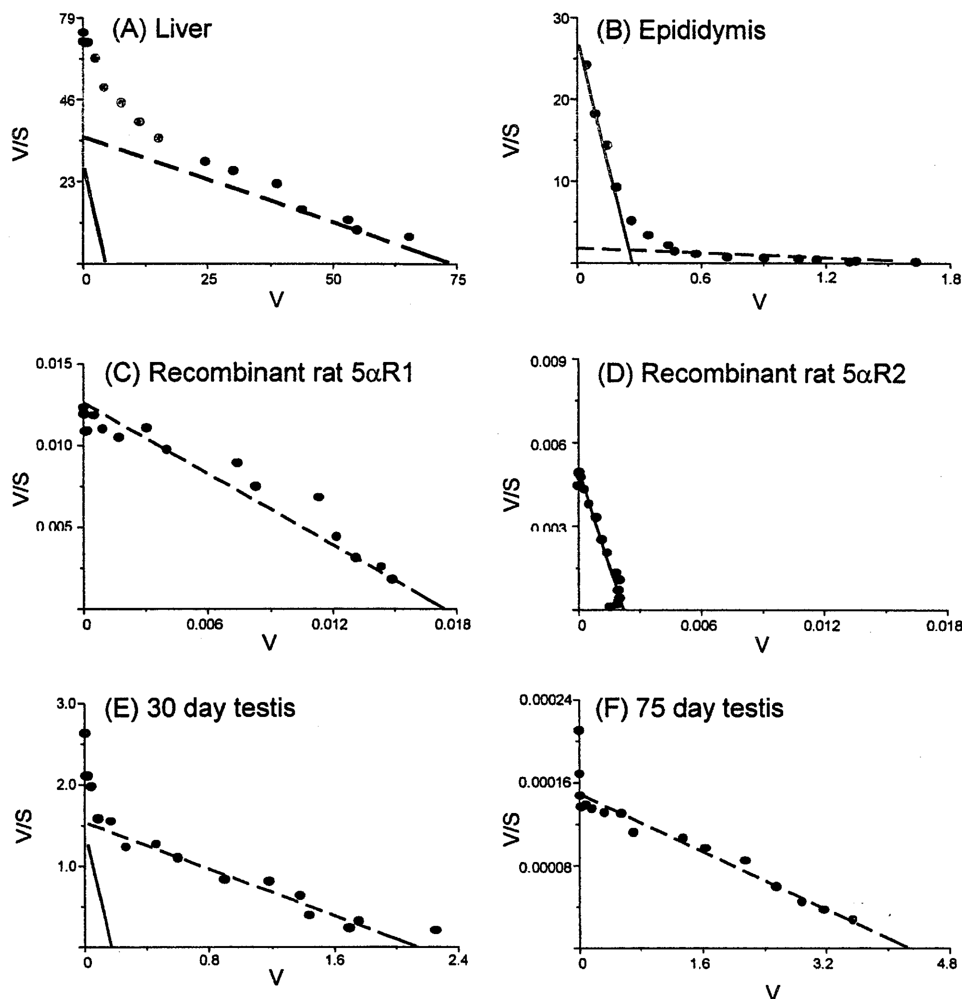

Fig. 1. Eadie–Scatchard plots for (A) liver, (B) epididymis, (C) recombinant rat 5α-R1, (D) recombinant 5α-R2, (E) 30 day testis and (F) 75 day testis. These graphs are representative of three separate experiments. Enzyme parameters (K_m, V_{max}, and K_m/V_{max}) for these analyses are presented in Table 1. Enzyme activity is presented as fmol DHT + 3α-Adiol per min per mg protein.

Table 1
Enzyme characteristics (K_m, V_{max}, and V_{max}/K_m ratio) for 5α-reductase activity in 30- and 75-day rat testis, epididymis, liver and recombinant rat 5αR Type 1 (5αR1) and Type 2 (5αR2) at pH 7.0[a]

Rat tissue/cells	5αR1			5αR2		
	K_m (μM)	V_{max} (fmol/min per mg protein)	V_{max}/K_m ($\times 10^{-12}$)	K_m (μM)	V_{max} (fmol/min per mg protein)	V_{max}/K_m ($\times 10^{-12}$)
30-day Rat testis	2.25 ± 0.96	3.73 ± 1.44	1.66	0.13 ± 0.09	0.26 ± 0.2	1.98
75-day Rat testis	3.06 ± 0.97	0.36 ± 0.14	0.12	ND	ND	
Rat epididymis	2.82 ± 1.39	1.35 ± 0.11	0.48	0.03 ± 0.03	0.35 ± 0.16	11.8
Rat liver (male)	2.41 ± 0.66	104 ± 21.6	43.2	0.11 ± 0.03	4.74 ± 0.86	43.9
Recombinant rat 5αR	1.64 ± 0.40	13.7 ± 4.84	8.32	0.27 ± 0.02*	3.16 ± 2.17	11.9

[a] Details of enzyme assays and analysis are presented in the Section 2. Values are the mean ± S.D. from three separate experiments. ND denotes nondetectable and * denotes enzyme kinetics performed at pH 5.0.

The pH activity profile for 5αR1 in rat liver (Fig. 2A) showed a broad neutral pH range (pH 5.5–7.5). The corresponding pH profile for 5αR2 in rat epididymis had an acidic pH optimum at pH 5.0 (Fig. 2A).

The 5αR pH activity profiles for 30 and 75-day testis (Fig. 2B) show similar patterns of activity across a broad neutral pH range (pH 5.5–7.5), although the activity at day 30 was approximately 40-fold higher than at day 75. Differences in activity at pH 5.0 suggest higher levels of 5αR2 in testis extracts from 30 day compared with 75 day rats. The activity of 5αR2 at pH 5.0, in the presence of 5αR1 activity, can be determined provided the contribution of 5αR1 activity at pH 5.0 is established.

3.3. Measurement of 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1

To determine the enzyme activity at pH 5.0 that can be attributed to 5αR1, rec 5αR1 was measured at pH 5.0 in the absence of any 5αR2 activity. Rec 5αR1 was transiently expressed in COS-7 cells, which expressed no endogenous 5αR activity (Table 2). The proportion of activity at pH 5.0 compared with pH 7.0 (Table 2) was determined to be 0.124 ± 0.014 ($n = 14$).

Given that the contribution of 5αR1 to 5αR activity at pH 5.0 was established, the following formula for measuring 5αR2 activity in the presence of 5αR1 was devised '5αR2 Activity = [(pH 5.0/7.0 sample) − 0.124] × [pH 7.0 sample]'.

3.4. Validation of assay method for measuring 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1

The assay was validated using the following criteria.

1. The sensitivity of the 5αR2 assay was defined as the activity calculated at two times the S.D. above the pH 5.0/7.0 ratio for rec 5αR1 (sensitivity, 0.152; Table 2). Tissue samples with a pH 5.0/7.0 ratio of less than or equal to this value were defined as nondetectable and given an activity equal to this sensitivity value.

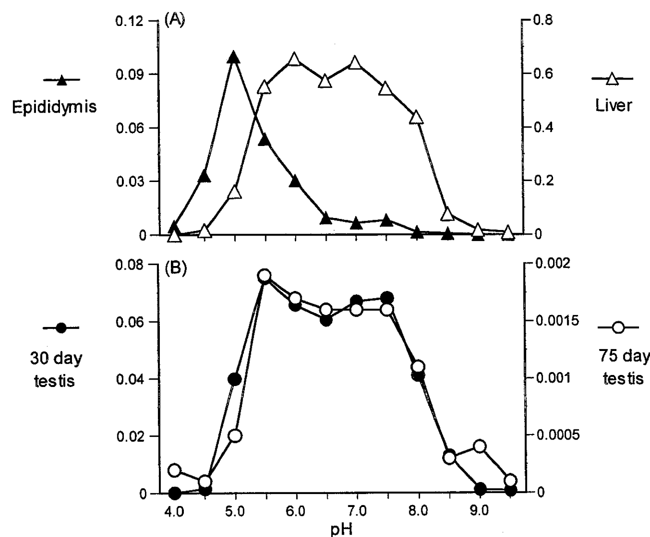

Fig. 2. 5α-Reductase activity (μmol DHT + 3α-Adiol per min per mg protein) as a function of pH for (A) 75-day epididymis (▲) and liver (△), and (B) 30 (●) and 75- (○) day rat testis. Note the different scales on the two y-axes for each graph.

Table 2
Recombinant rat 5αR1 enzyme activity at pH 5.0 and 7.0[a]

Expression plasmid	Recombinant 5αR1 activity (fmol DHT + 3α-Adiol per min per mg protein)		
	pH 5.0	pH 7.0	pH 5.0/7.0 ratio
Vector	<0.3[b]	<0.3[b]	–
Recombinant 5αR1	35.3 ± 10.2	283 ± 56.3	0.124 ± 0.014

[a] Values are mean ± S.D. from five separate transfections and a total of 14 different measurements.

[b] At the detection limits of the assay (~0.2% conversion of substrate).

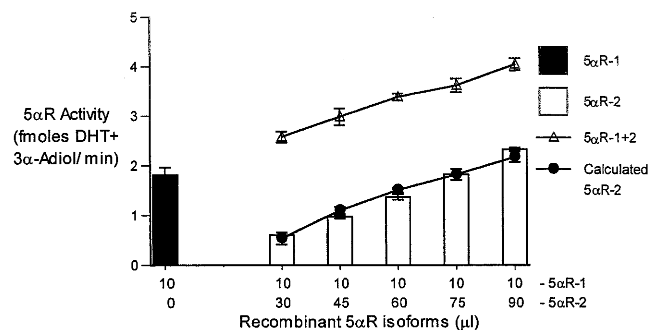

Fig. 3. Measurement of 5αR2 activity in the presence of 5αR1 activity. Increasing amounts of recombinant rat 5αR2 were measured alone (open bars) or in combination with a constant amount of recombinant 5αR1 (open triangles). The levels of 5αR2 were determined by the proposed 5αR2 assay (closed circles). Enzyme activity is presented as fmol DHT + 3α-Adiol per min.

2. To determine the accuracy of the assay, increasing concentrations of recombinant rat 5αR2 (rec 5αR2) were measured in the presence of a fixed concentration of rec 5αR1. A representative experiment (Fig. 3A) demonstrated that by correcting for the overlap of 5αR1 activity at pH 5.0 with the formula mentioned above, 5αR2 can be accurately measured. The correlation of added rec 5αR2 activity versus calculated 5αR2 activity from three separate experiments (data not shown) gave a linear response, with a correlation coefficient of $r = 0.98$ and a recovery of 99.3 ± 14.7% ($n = 12$).
3. The within-assay variation was assessed from the average CV within replicates for samples assayed and was 4.0% ($n = 56$). The between-assay variation was assessed from the reproducibility of measurement of a testicular extract which was included as a QC in every assay and was 3.6% ($n = 14$).

3.5. Measuring 5α-reductase Type 1 and 2 activity in rat testis extracts

5αR1 activity at pH 7.0 and 5αR2 activity at pH 5.0 using the method described above were determined in 10 000 × g testicular supernatants from rats aged 30, 75 and 147 days (Table 3). The pH 5.0/7.0 ratio is also shown for comparison, as this method is often used as an estimate of 5αR2 activity [26]. 5αR1 activity declined with age to reach < 1% the levels of day 30 at 147 days. The corresponding values for 5αR2 showed a similar decline with age to reach values < 2% that of day 30 by 147 days. 5αR2 levels in three of the six animals at day 75 were nondetectable and have been given an activity value at the level of detection of the assay. The levels of 5αR2 at 147 days were readily detectable, despite the similar or even lower pH 5.0 activity compared with 75 days. This is due to the fact that there is lower 5αR1 activity at day 147, resulting in a higher pH 5.0/7.0 ratio at 147 days (0.335) compared with 75 days (0.136–0.187; Table 3).

4. Discussion

Several studies have examined 5αR1 activity in rat testis, focusing mainly on the immature rat testis where DHT levels are high and thus suggested to play a role in androgen physiology [14–20]. To the best of our knowledge there are no studies that have investigated 5αR2 activity in rat testis. With the intention of investigating the regulation of 5αR, the type of 5αR activity expressed in rat testis at various ages was first investigated. Enzyme kinetics was initially used to investigate 5αR isoform activity in rat testis. Both isoforms were found to be expressed at day 30, and the K_m values obtained for the isoforms were comparable to those obtained for the liver, epididymis and rec 5αR1 and rec 5αR2. At 75 days, however, only 5αR1 activity was detected. Comparison of V_{max} values at day 30 showed that 5αR1 was quantitatively (> 93%) the predominant isoform. The inability to detect 5αR2 at day 75 by enzyme kinetic analysis is attributed to the lack of sensitivity of this method to differentiate between the 5αR isoforms when 5αR2 levels are very low.

A new method was developed in order to measure low levels of 5αR2 activity in the presence of high 5αR1 levels in rat testis. The pH 5.0/7.0 ratio has commonly been used as a relative measure for 5αR2 activity when a sample expresses both 5αR isoforms [26]. This measurement, however, is an estimate and thus is not

Table 3
Measurement of 5αR1 and 5αR2 activities in 30, 75, and 147-days rat testis (pmol DHT + 3α-Adiol per min per mg protein)[a]

Rat testis	*N*	5αR1	5αR2		
		pH 7.0	pH 5.0	pH 5.0/7.0 ratio	Calculated 5αR2
30 days	11	46.1 ± 9.67	15.4 ± 3.23	0.339 ± 0.069	3.44 ± 1.49
75 days	3	2.70 ± 1.54	0.49 ± 0.24	0.187 ± 0.013	0.16 ± 0.04
	3	1.90 ± 0.16	0.26 ± 0.03	0.136 ± 0.006	0.05 ± 0.01*
147 days	7	0.25 ± 0.05	0.08 ± 0.03	0.335 ± 0.112	0.06 ± 0.02

[a] Mean ± S.D.;*, Denotes below the sensitivity of the assay; pH 5.0/7.0 ratio <0.152.

quantitative. In order to measure 5αR2 at pH 5.0 in the presence of 5αR1 activity, the contribution of 5αR1 to the activity at pH 5.0 needs to be known. Therefore, the amount of 5αR1 activity at pH 5.0 and 7.0 was measured in COS-7 cells transiently transfected with a cDNA encoding rat 5αR1. These cells did not contain endogenous 5αR activity. The pH 5.0/7.0 ratio for rec rat 5αR1 was 0.124 ± 0.014 (i.e. 12.4% of the activity detected at pH 5.0 can be attributed to 5αR1 activity). Thus when pH 5.0 activity is used to measure 5αR2 activity, the 12.4% attributed to 5αR1 must be corrected to accurately measure 5αR2. Hence, a formula was devised to take into account the pH 5.0/7.0 ratio of rec 5αR1, removing the activity due to 5αR1.

To test this formula a constant amount of rec 5αR1 was combined with varying known concentrations of rec 5αR2. These samples were assayed at pH 5.0 and 7.0 and the formula used to measure 5αR2 activity. These values were then compared with the actual amount of rec 5αR2 added, and a linear correlation was obtained, indicating that this method allowed an accurate determination of 5αR2 activity in the presence of 5αR1.

This method was then applied to assay 5αR1 and 5αR2 activities in rat testis at days 30, 75 and 147. 5αR1 activity declined with increasing age. This is consistent with previous findings that showed a peak expression of 5αR1 activity [13,14,17,18] and mRNA [4] at day 30, with very low levels of 5αR1 activity at day 75 [17,18]. In our study, 5αR2 activity also showed a similar pattern of expression with 5αR1. 5αR2 was detected in the testis of some animals at day 75, which was previously not detectable by enzyme kinetics.

Based on the V_{max} values (i.e. enzyme concentration) there is approximately three times more 5αR1 in the epididymis than there is 5αR2. The V_{max}/K_m ratio is an estimation of the potential in vivo isoform activity when the endogenous T concentration is much lower than the K_m value [26,27]. Based on V_{max}/K_m values the higher efficiency ratio for 5αR2 in the epididymis compared with 5αR1 (25-fold) suggests that even though 5αR1 is present in higher concentrations, the potential in vivo activity at pH 7.0 could be attributed mainly to 5αR2. Similarly in rat testis at day 30, even though the V_{max} indicated that there is approximately 15 times higher concentration of 5αR1 than there is 5αR2, the potential in vivo activity could be attributed equally to both isoforms.

In summary, by removing the contribution of 5αR1 activity at pH 5.0, 5αR2 activity can be measured accurately in the presence of high levels of 5αR1 activity. This method was used to examine 5αR isoform expression in rat testis to show that 5αR1 is the predominant isoform expressed, and that the activity of both 5αR isoforms decline with age. The sensitivity and specificity of this assay has allowed the investigation of testicular 5αR isoforms at low expression levels (> 70 days). The regulation of the 5αR isoforms in the testis is central to our understanding of their role in spermatogenesis, especially in the contraceptive setting. Current studies are focusing on the hormonal regulation of the 5αR isoforms in rat testis by measuring enzyme activity and using PCR methods to quantitate mRNA levels.

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References

[1] R.J. Moore, J.D. Wilson, Localization of the reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase in the nuclear membrane of the rat ventral prostate, J. Biol. Chem. 247 (1972) 958–967.

[2] S. Andersson, D.W. Russell, Structural and biochemical properties of cloned and expressed human and rat steroid 5α-reductases, Proc. Natl. Acad. Sci. USA 87 (1990) 3640–3644.

[3] S. Andersson, D.M. Berman, E.P. Jenkins, D.W. Russell, Deletion of steroid 5α-reductase 2 gene in male pseudohermaphroditism, Nature 354 (1991) 159–161.

[4] K. Normington, D.W. Russell, Tissue distribution and kinetic characteristics of rat steroid 5α-reductase isozymes, J. Biol. Chem. 267 (1992) 19548–19554.

[5] E.P. Jenkins, S. Andersson, J.D. Imperato-McGinley, J.D. Wilson, D.W. Russell, Genetic and pharmacological evidence for more than one human steroid 5α-reductase, J. Clin. Invest. 89 (1992) 293–300.

[6] A.E. Thighpen, D.L. Davis, A. Milatovich, B.B. Mendonca, J. Imperato-McGinley, J.E. Griffin, U. Francke, D.W. Russell, Molecular genetics of steroid 5α-reductase 2 deficiency, J. Clin. Invest. 90 (1992) 799–809.

[7] P.B. Grino, J.E. James, J.D. Wilson, Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone, Endocrinology 126 (1990) 1165–1172.

[8] Z. Zhou, M.V. Lane, J.A. Kemppainen, F.S. French, E.M. Wilson, Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability, Mol. Endocrinol. 9 (1995) 208–218.

[9] L. O'Donnell, P.G. Stanton, N.G. Wreford, D.M. Robertson, R.I. McLachlan, Inhibition of 5α-reductase activity impairs the testosterone-dependent restoration of spermatogenesis in adult rats, Endocrinology 137 (1996) 2703–2710.

[10] L. O'Donnell, P.G. Stanton, N.G. Wreford, D.M. Robertson, R.I. McLachlan, Testosterone-dependent restoration of spermatogenesis in adult rats is impaired by a 5α-reductase inhibitor, J. Androl. 1999 (1999) 109–117.

[11] R.S. Viger, B. Robaire, Steady state steroid 5α-reductase messenger ribonucleic acid levels and immunocytochemical localization of the type 1 protein in the rat testis during postnatal development, Endocrinology 136 (1995) 5409–5415.
[12] Y. Folman, J.G. Sowell, K.B. Eik-Nes, The presence and formation of 5α-dihydrotestosterone in rat testes in vivo and in vitro, Endocrinology 91 (1972) 702–710.
[13] M.A. Rivarola, E.J. Podesta, H.E. Chemes, In vitro testosterone -^{14}C metabolism by rat seminiferous tubules at different stages of development: formation of 5α-androstan-diol, Endocrinology 91 (1972) 537–542.
[14] E.J. Podesta, M.A. Rivarola, Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development, Endocrinology 95 (1974) 455–461.
[15] J.H. Dorrington, I.B. Fritz, Cellular localization of 5α-reductase and 3α-hydroxysteroid dehydrogenase in the seminiferous tubule of the rat testis, Endocrinology 96 (1975) 879–889.
[16] Y. Folman, N. Ahmad, J.G. Sowell, K.B. Eik-Nes, Formation in vitro of 5α-dihydrotestosterone and other 5α-reduced metabolites of ^{3}H-testosterone by the seminiferous tubules and interstitial tissue from immature and mature rat testes, Endocrinology 92 (1973) 41–47.
[17] K. Matsumoto, M. Yamada, 5α-Reduction of testosterone in vitro by rat seminiferous tubules and whole testis at different stages of development, Endocrinology 93 (1973) 253–255.
[18] H.J. Van der Molen, J.A. Grootegoed, M.J. de Greef-Bijleveld, F.F.G. Rommerts, G.J. van der Vusse, Distribution of steroids, steroid production and steroid metabolizing enzymes in rat testis, in: F.S. French, V. Hansson, E.M. Ritzen, S.N. Nayfey (Eds.), Hormonal Regulation of Spermatogenesis, vol. II, Plenum Press, New York, 1975, pp. 3–23.
[19] K. Yoshizaki, K. Matsumoto, L.T. Samuels, Localization of Δ^{4}-5α-reductase in immature rat testes, Endocrinology 102 (1978) 918–925.
[20] M.A. Rivarola, E.J. Podesta, Metabolism of testosterone-^{14}C by seminiferous tubules of mature rats: formation of 5α-androstan-3α-diol-^{14}C, Endocrinology 90 (1972) 618–623.
[21] J.H. Dorrington, I.B. Fritz, Metabolism of testosterone by preparations from the rat testis, Biochem. Biophys. Res. Commun. 54 (1973) 1425–1431.
[22] M.A. Drodowsky, M. Tence, I. Barral, Biosynthesis and metabolism of testosterone in isolated germ cells of the rat and the ram and in the Sertoli cells of the rat, in: F.S. French, V. Hansson, E.M. Ritzen, S.N. Nayfey (Eds.), Hormonal Regulation of Spermatogenesis, vol. II, Plenum Press, New York, 1975, pp. 109–121.
[23] E.P. Murono, A.H. Payne, Testicular maturation in the rat. In vivo effect of gonadotrophins on steroidogenic enzymes in the hypophysectomized immature rat, Biol. Reprod. 20 (1979) 911–917.
[24] I.H. Segal, Enzyme Kinetics, Wiley, New York, 1975, pp. 214–218.
[25] C.M. Gorman, D.R. Cies, G. McCray, Transient production of proteins using an adenovirus transformed cell line, DNA Protein Eng. Tech. 2 (1990) 3–10.
[26] A.E. Thighpen, K.M. Cala, D.W. Russell, Characterization of Chinese hamster ovary, J. Biol. Chem. 268 (1993) 17404–17412.
[27] M. Krieg, W. Bartsch, M. Thomsen, K.D. Voigt, Androgens and estrogens: their interaction with stroma and epithelium of human benign prostatic hyperplasia and normal prostate, J. Steroid Biochem. (1983) 155-161.