Differential regulation of rat testicular 5 α -reductase type 1 and 2 isoforms by testosterone and FSH

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Abstract

Testosterone is metabolised to the more potent androgen, dihydrotestosterone, by the 5 α -reductase (5 α R) enzyme. We previously showed that 5 α -reduced androgens are important for maintaining androgen action on rat spermatogenesis when testicular testosterone concentrations are reduced. This study investigated expression and activity of the 5 α R isoforms, type 1 (5 α R-1) and type 2 (5 α R-2), in the rat during hormone manipulation in order to understand the factors that regulate the testicular concentration of 5 α R and testicular 5 α -reduced androgen biosynthesis. Testicular 5 α R-1 and 5 α R-2 mRNA and enzyme activity were measured by real-time PCR and specific enzyme assays respectively. Hormone levels were first suppressed using two models of gonadotrophin suppression: testosterone and oestradiol treatment (LH/testosterone deficiency) or GnRH immunisation (LH/testosterone and FSH deficiency). Hormones were then either restored or suppressed for 6 days by a variety of hormonal treatments. 5 α R-1 mRNA and enzyme activity increased when

testosterone was suppressed, yet restoration of testosterone decreased 5 α R-1 mRNA and enzyme activity, suggesting that testosterone negatively regulates 5 α R-1. Suppression of FSH decreased 5 α R-1 mRNA yet FSH administration increased 5 α R-1 mRNA, but no changes in 5 α R-1 activity were observed within the 6 day period. In contrast to 5 α R-1, testosterone did not affect the testicular concentration of 5 α R-2 mRNA or activity, but there was evidence for modulation of 5 α R-2 activity by FSH. Measurement of testicular androgens revealed that 5 α R-1 was primarily responsible for the production of 5 α -reduced metabolites. It is concluded that the 5 α R isoforms in rat testis are differentially regulated by testosterone and FSH: testosterone negatively regulated 5 α R-1 mRNA and enzyme activity but had no effect on 5 α R-2, whereas FSH positively regulated 5 α R-1 mRNA and appeared to regulate 5 α R-2.

Journal of Endocrinology (2003) **176**, 393–403

Introduction

Testosterone is the predominant androgen involved in the regulation of normal spermatogenesis in the rat due to its high intratesticular concentration (Wright & Frankel 1979). The process of spermatogenesis is critically dependent on testosterone and the amount of sperm produced is dose-responsive to the level of testosterone in the testis (Awoniyi *et al.* 1989, McLachlan *et al.* 1994). The 5 α -reduction of testosterone to the more potent androgen, 5 α -dihydrotestosterone (DHT), is postulated to be important for maintaining spermatogenesis when intratesticular testosterone concentrations are low (Anderson *et al.* 1996, O'Donnell *et al.* 1996, 1999).

Testosterone has been the target for suppressing spermatogenesis in numerous contraceptive trials. In both primates (Narula *et al.* 2002) and men (McLachlan *et al.* 2002), gonadotrophin suppression by exogenous testosterone administration caused a marked reduction in testicular

testosterone levels yet maintenance of 5 α -reduced androgen levels. It is thought that maintenance of testicular 5 α -reduced androgen levels may be responsible for continued low levels of sperm production in the ~30% of normal men whose sperm counts do not fall to zero (azoospermia) after testosterone contraceptive treatment (Anderson *et al.* 1996, 1997). In the rat model of spermatogenic suppression, administration of a 5 α -reductase (5 α R) inhibitor to prevent DHT production suppressed spermatogenesis (O'Donnell *et al.* 1996, 1999). Furthermore, administration of the androgen receptor (AR) antagonist flutamide during spermatogenic suppression in the rat increased the production of 5 α -reduced metabolites in the testis, suggesting that 5 α R may be hormonally regulated (O'Donnell *et al.* 1999). Taken together, these studies suggest that testicular 5 α R may be regulated by gonadotrophins and/or testosterone and that 5 α R activity is important for determining the amount of bioactive androgen in the testis.

Two isoforms of 5 α R, type 1 (5 α R-1) and type 2 (5 α R-2), have been cloned in rats and humans (Andersson & Russell 1990, Normington & Russell 1992). These isoforms are products of different genes and possess different biochemical properties: 5 α R-1 has a micromolar affinity for steroid substrates with peak activity extending over a broad neutral pH range, whereas 5 α R-2 has a nanomolar affinity for steroid substrates with peak activity at pH 5.0 (Andersson & Russell 1990). The 5 α R isoforms have not been extensively investigated in rat testis. Viger & Robaire (1995) used Northern blots to show expression of 5 α R-1 in postnatal and adult rat testis, whereas 5 α R-2 was not detected. In contrast, Normington & Russell (1992) used RNA blot hybridisation to show that 5 α R-2 was the predominant isoform expressed in adult rat testis. The development of enzyme activity assays for measuring rat testicular 5 α R-1 and 5 α R-2 activity in the presence of high 5 α R-1 levels revealed that 5 α R-1 was the predominant isoform in the pubertal and adult rat testis, with both 5 α R isoforms being lower in adult testis (Pratis *et al.* 2000).

Understanding the regulation of the testicular 5 α R isoforms is important, given their central role in androgen biosynthesis, and thus the potential for influencing sperm production. Thus, the aim of this study was to investigate the hormonal regulation of 5 α R-1 and 5 α R-2 mRNA and enzyme activity in adult rat testis, using two *in vivo* models of luteinizing hormone (LH)/testosterone and/or follicle-stimulating hormone (FSH) suppression. LH/testicular testosterone was suppressed with testosterone and oestradiol implants, and LH/testosterone and FSH was suppressed by active immunisation of rats against gonadotrophin-releasing hormone (GnRH). In both models, the AR antagonist, flutamide, was used to suppress residual levels of androgens, and residual FSH levels were suppressed with an FSH antiserum. Testosterone was partially or fully restored with high doses of testosterone (using implants) and human chorionic gonadotrophin (hCG) respectively. FSH action was restored by administering recombinant human FSH (rhFSH). The 5 α R-1 and 5 α R-2 mRNA levels were quantified by real-time PCR and enzyme activities were measured with an *in vitro* activity assay.

Materials and Methods

Reagents, hormones and drugs

Organic solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and thin layer chromatography plates (ITLC-SA) were obtained from Gelman Sciences (NSW, Australia). Non-radioactive steroids were purchased from Sigma (St Louis, MO, USA), radioactive steroids were purchased from Du Pont-New England Nuclear (Boston, MA, USA). [4-¹⁴C]-3 α -androstenediol (3 α -Adiol) was prepared as described previously (Pratis *et al.* 2000). Flutamide (Sigma) was dissolved in methylene

chloride and added to peanut oil (methylene chloride:oil, 1:2 v/v). The methylene chloride was evaporated by bubbling with nitrogen and warming the solution to 37 °C. The FSH.Ab was an immunoglobulin fraction of anti-rat FSH antibody raised in sheep (FSH.Ab #1792; Meachem *et al.* 1998). rhFSH (Gonal-F) was obtained from Serono (Sydney, Australia). hCG (Pregnyl) was obtained from Organon (Sydney, Australia).

Animals

Adult male Sprague-Dawley rats were obtained from the Monash Central Animal House (Clayton, Australia) and housed under a 12 h light:12 h darkness cycle with free access to food and water. Animals were killed by CO₂ asphyxiation, blood was removed by cardiac puncture and the testes quickly removed, trimmed of fat, weighed and frozen in liquid nitrogen. Blood was stored at -20 °C and testes were stored at -70 °C until required. These studies were approved by the Monash Medical Centre Animal Ethics Committee.

Experimental design

Two *in vivo* models of gonadotrophin suppression were used to investigate the regulation of 5 α R by testosterone and FSH. All animals except controls received two injections, one in oil and the other in normal rat serum (NRS). All preparations (except flutamide) were prepared in 15% NRS and filtered through a 0.2 μ m filter (Millipore, Bedford, MA, USA). Vehicle oil injections were similarly prepared except the drug/hormone was omitted.

Testosterone and oestradiol suppression model

Testosterone (3 cm) and oestradiol (0.4 cm) (TE) implants were prepared by filling medical-grade polydimethylsiloxane Silastic tubing (outer diameter (o.d.) 3.18 mm; inner diameter (i.d.) 1.98 mm; Dow-Corning, Midland, MI, USA) with testosterone and oestradiol respectively. Twenty-four centimetre testosterone (T24) implants were prepared using 3 $\times$ 8 cm testosterone implants. Animals were anaesthetised by ether inhalation (as approved by Monash Medical Centre Animal Ethics Committee) before implantation and the steroid implants placed s.c. along the animals' dorsal surface. Six groups of adult rats received TE implants for 61 days (Table 1). Following TE suppression and during the treatment phase, three groups retained the TE implants while in the other three groups the TE implants were replaced with T24 implants. TE- and T24-treated animals received vehicle, flutamide (20 mg/kg per day), or FSH.Ab (2 mg/kg per day). Controls did not receive TE implants or any treatment during the treatment phase.

GnRH immunisation model Animals were actively immunised against GnRH with a proprietary GnRH

Table 1 TE suppression model. Animals received T24 implants on day 2 of the treatment phase

Group	<i>n</i>	Suppression phase (8 weeks)	Treatment phase (6 days)
1	8	–	Untreated control
2	8	TE	TE+vehicle
3	8	TE	TE+flutamide
4	7	TE	TE+FSH.Ab
5	8	TE	T24+vehicle
6	8	TE	T24+flutamide
7	8	TE	T24+FSH.Ab

immunogen preparation (BA-1666-4; Biotech, Roseville, NSW, Australia) incorporating an adjuvant free of myobacterial components (Stewart *et al.* 1992). Animals received 100 μ l GnRH immunogen every 4 weeks at a single s.c. site over a 12-week period as previously described (McLachlan *et al.* 1994, 1995, Meachem *et al.* 1998). Five groups of adult rats received GnRH immunisations (Table 2). During the treatment phase animals received either vehicle, FSH.Ab (2 mg/kg per day), or rhFSH (25 IU/kg per day), and two GnRH-immunised groups were treated with hCG (2.5 IU/kg per day). Controls did not receive the immunogen or any treatment during the treatment phase.

5 α R mRNA

Total RNA was extracted from rat testis using the Qiagen RNeasy kit (Qiagen, VIC, Australia) and treated with DNase (DNA-free; Ambion, Austin, TX, USA) to remove any contaminating DNAs. Extracted RNA (2 μ g) was reverse transcribed with either 100 ng random primer (for 5 α R-1 mRNA) or 125 ng oligo (dT)_{12–18} (for 5 α R-2 mRNA) at 42 °C for 50 min using 50 U of Expand reverse transcriptase (Roche Molecular, Mannheim, Germany). Following reverse transcription, the reaction was terminated by heating at 70 °C for 15 min. The cDNAs were stored at 4 °C until required for use in real-time PCR.

5 α R mRNA was quantified by real-time RT-PCR amplification in a LightCycler (Roche) instrument (Bustin

2000) using SYBR Green I fluorescence detection of amplified products. The forward (5'-TCC TGG TCA CCT TTG TCT TGG C-3') and reverse (5'-GTT TCC CCT GGT TTT CTC AGA TTC-3') primers for 5 α R-1 were designed to produce a 128 bp amplicon. The forward (5'-ACA TCC ACA GTG ACT ACA CCC TGC-3') and reverse (5'-TCC ATT CAA TAA TCT CGC CCA G-3') primers for 5 α R-2 were designed to produce a 207 bp amplicon. A 20 μ l PCR reaction was used and included PCR grade water (10.8 μ l), 5 mmol/l magnesium chloride, specific reverse and forward primers (0.5 μ mol/l final concentration for 5 α R-1 and 0.375 μ mol/l final concentration for 5 α R-2), SYBR Green dye (2 μ l) and reverse-transcribed cDNAs (2 μ l, diluted 1:10). An initial denaturation step of 10 min was used for both 5 α R-1 and 5 α R-2. PCR amplification for 5 α R-1 consisted of 38 cycles at 95 °C for 15 s (denaturation), 55 °C for 5 s (annealing), and 72 °C for 10 s (elongation). PCR amplification for 5 α R-2 consisted of 40 cycles at 95 °C for 15 s (denaturation), 62 °C for 5 s (annealing), and 72 °C for 10 s (elongation). The DNA standards were highly purified and sequence-verified cDNAs, which were identical to the real-time PCR products to ensure equal amplification efficiency between standards and PCR products. For each quantification, standard curves consisting of twofold dilutions of the appropriate 5 α R cDNA were included, and these ranged between 1 and 0.0625 fg for 5 α R-1 and 0.2 and 0.0125 fg for 5 α R-2. Following PCR, melting curve analysis was performed on the amplified product to ensure that only specific PCR amplicons were obtained and quantified. This was also confirmed by nucleotide sequencing. The mRNA data obtained from the LightCycler were normalised against 28S ribosomal RNA, which was quantified by electrophoresing equivalent RNA on an agarose gel and the 28S ribosomal RNA fluorescence measured using a CCD camera with Quantity One 5.1 software (Bio-Rad, NSW, Australia). All measurements were done within the linear range under non-saturating pixel conditions. The electrophoresed RNA was also used to confirm the integrity of all RNAs used in the real-time PCR analysis. All samples were measured in eight separate assays. Between-assay variation as determined from the reproducibility of a quality control sample was 8.9% for 5 α R-1 and 8.8% for 5 α R-2. 5 α R-1 and 5 α R-2 mRNA was measured in all animals except for the T24-treated animals in the TE suppression study due to insufficient sample amounts.

5 α R enzyme activity

The remainder of the left testis was homogenised in 0.25 mol/l sucrose, centrifuged at 10 000 *g* for 20 min and the supernatant used for measuring 5 α R-1 and 5 α R-2 enzyme activity with enzyme assays validated previously (Pratis *et al.* 2000). 5 α R-1 activity was measured at pH 7.0

Table 2 GnRH immunisation (GnRH_{imm}) model

Group	<i>n</i>	Suppression phase (12 weeks)	Treatment phase (6 days)
1	8	–	Untreated control
2	8	GnRH _{imm}	GnRH _{imm} +vehicle
3	8	GnRH _{imm}	GnRH _{imm} +FSH.Ab
4	8	GnRH _{imm}	GnRH _{imm} +rhFSH
5	8	GnRH _{imm}	GnRH _{imm} +hCG
6	8	GnRH _{imm}	GnRH _{imm} +hCG+FSH.Ab

and 5 α R-2 activity was determined at pH 5.0. The contribution of the 5 α R-1 isoform at pH 5.0 in the 5 α R-2 activity measured (i.e. overlap) was determined and subtracted to give specific 5 α R-2 enzyme activity.

Briefly, the two assays measure the conversion of [3 H]testosterone to [3 H][DHT]+[3 H]3 α -Adiol in 0.1 mol/l Tris-citrate buffer at their respective pH, followed by steroid extraction with toluene:ether (1:5), steroid separation with TLC, and steroid quantification by scintillation β -counting. 5 α R activity was expressed as the amount of DHT and 3 α -Adiol produced after corrections for procedural losses, determined using [14 C]steroids (testosterone, DHT and 3 α -Adiol) as internal standards. The blank activity for the 5 α R assay (in the absence of testicular supernatant) was 0.010 ± 0.003 pmol DHT+3 α -Adiol/min ($n=6$), which corresponded to less than 0.12% conversion of substrate.

LH, FSH and testicular steroids

Serum LH was measured by immunofluorometric assay (Haavisto *et al.* 1993) using rat LH (rLHRP-3; NIDDK, Bethesda, MD, USA) as a standard. All samples were assayed in two assays with a detection limit of 0.02 ng/ml. Serum FSH was measured by RIA using iodinated rat FSH (rFSH 17; NIDDK) as tracer, rFSH antiserum (anti-rFSH S11; NIDDK), and rFSH RP2 as standard. All samples were assayed in two assays with a detection limit of 1.5 ng/ml.

The right testis from each rat was kept for measurement of testicular steroids. Testicular testosterone, DHT and 3 α -Adiol were extracted with acetonitrile, separated by HPLC and measured by RIA, as described previously (O'Donnell *et al.* 1996). The within-assay variations for testosterone, DHT and 3 α -Adiol were 8, 13 and 12% respectively, and the between-assay variations were 8, 3 and 8% respectively ($n=6$ assays).

Statistics

Differences between groups (data with equal variances, as assessed by Bartlett's test) were assessed by ANOVA and independent *t*-tests. Data with unequal variances were assessed by the Kruskal-Wallis test and Dunn's multiple comparison test between selected treatment groups. All data were expressed as means $\pm$ S.E.M. ($n=7$ or 8) and considered significant when $P<0.05$ was achieved.

Results

TE suppression model

Serum LH and FSH Serum LH was suppressed ($P<0.05$) in all TE-treated groups compared with controls and administration of flutamide to TE- and T24-treated groups increased ($P<0.05$) LH levels above corresponding

Table 3 TE suppression model: serum LH and FSH, means $\pm$ S.E.M. (see Table 1 and Methods section for further details)

	LH (ng/ml)	FSH (ng/ml)
Treatment group		
Untreated control	1.15 $\pm$ 0.15	3.65 $\pm$ 0.12
TE+vehicle	0.12 $\pm$ 0.02*	3.06 $\pm$ 0.11
TE+flutamide	0.69 $\pm$ 0.16*†	6.47 $\pm$ 0.84*†
TE+FSH.Ab	0.08 $\pm$ 0.01*	NA
T24+vehicle	0.11 $\pm$ 0.02*	4.02 $\pm$ 0.33
T24+flutamide	0.41 $\pm$ 0.09*†	5.59 $\pm$ 0.52
T24+FSH.Ab	0.08 $\pm$ 0.01*	NA

NA denotes not assayed due to interference of FSH.Ab in the FSH RIA.

*Significant difference compared with untreated control. †Significant difference compared with corresponding TE+vehicle or T24+vehicle.

vehicle-treated animals (Table 3). Serum FSH was not affected by TE or T24 treatment; however, TE+flutamide treatment significantly increased FSH above TE+vehicle (Table 3).

Testicular 5 α R-1 and 5 α R-2 mRNA and enzyme activity

There was no difference in the housekeeping gene 28S ribosomal RNA between any of the treatment groups (Fig. 1A). 5 α R-1 mRNA (Fig. 1B) and enzyme activity (Fig. 1C) in TE-suppressed animals increased ($P<0.05$) above controls levels, while administration of flutamide to TE-suppressed rats further increased 5 α R-1 mRNA and enzyme activity levels ($P<0.05$, Fig. 1B and C). T24 treatment did not change 5 α R-1 enzyme activity compared with TE+vehicle treatment; however, T24+flutamide treatment increased ($P<0.05$) 5 α R-1 enzyme activity above the T24+vehicle group (Fig. 1C). TE+FSH.Ab treatment decreased ($P<0.05$) 5 α R-1 mRNA compared with TE+vehicle treatment but did not change 5 α R-1 enzyme activity.

5 α R-2 mRNA was suppressed below control by TE suppression ($P<0.05$), while acute FSH.Ab treatment further suppressed 5 α R-2 mRNA levels by ~ 4.5 -fold ($P<0.05$, Fig. 1D). 5 α R-2 enzyme activity was not affected by these treatments (Fig. 1E).

Testicular steroids TE treatment suppressed testicular testosterone to $<3\%$ of control levels ($P<0.05$, Fig. 2A), whereas DHT and 3 α -Adiol (Fig. 2B and C respectively) were suppressed to 26 and 53% of controls ($P<0.05$) respectively. Flutamide administration significantly increased DHT and 3 α -Adiol levels compared with TE treatment, in the absence of a significant increase in testosterone. Acute FSH.Ab administration did not significantly alter any of the androgens. TE suppression significantly increased the conversion of testosterone to 5 α -reduced metabolites (DHT+3 α -Adiol/testosterone, Fig. 2D). Co-administration of flutamide significantly ($P<0.05$) increased this conversion while FSH had no effect.

GnRH immunisation model

Serum LH and FSH GnRH immunisation significantly suppressed both serum LH and FSH below control levels and administration of hCG to GnRH-immunised animals partially restored ($P < 0.05$) serum FSH (Table 4). Administration of hCG to GnRH-immunised animals partially restored serum FSH, presumably as a consequence of a rise in circulating testosterone, which stimulates FSH in a GnRH-independent manner (Rea *et al.* 1986, Bhasin *et al.* 1987).

Testicular 5 α R-1 and 5 α R-2 mRNA and enzyme activity There was no difference in the housekeeping gene 28S ribosomal RNA between any of the treatment groups (Fig 3A). 5 α R-1 mRNA levels (Fig. 3B) decreased to 49% of control following GnRH immunisation ($P < 0.05$), which returned to control levels following treatment with rhFSH or hCG ($P < 0.05$). The hCG-induced increase in 5 α R-1 mRNA was inhibited ($P < 0.05$) by the co-administration of FSH.Ab. 5 α R-1 enzyme activity (Fig. 3C) was significantly increased above control by GnRH immunisation, and this increase was not affected by administration of FSH.Ab or rhFSH. However, hCG treatment, with or without FSH.Ab treatment, suppressed ($P < 0.05$) 5 α R-1 enzyme activity below the corresponding GnRH immunisation+vehicle treatment.

5 α R-2 mRNA (Fig. 3D) appeared to be lower in GnRH-immunised animals; however, this was not significant. Co-administration of FSH.Ab with hCG treatment increased 5 α R-2 mRNA compared with corresponding vehicle-treated animals ($P < 0.05$). 5 α R-2 enzyme activity (Fig. 3E) was suppressed ($P < 0.05$) by GnRH immunisation and restored to control levels with rhFSH or hCG treatment ($P < 0.05$). The hCG-induced increase in 5 α R-2 enzyme activity was prevented by co-administration of FSH.Ab ($P < 0.05$).

Testicular steroids GnRH immunisation suppressed testicular testosterone to $< 2\%$ of control ($P < 0.05$, Fig. 4A). hCG treatment (with or without FSH.Ab) significantly increased testosterone levels. Testicular DHT (Fig. 4B) and 3 α -Adiol (Fig. 4C) in GnRH-immunised animals did not change with rhFSH or FSH.Ab treatment.

GnRH immunisation increased the conversion of testosterone to 5 α -reduced metabolites (DHT+3 α -Adiol/

Figure 1 5 α R-1 and 5 α R-2 mRNA (B and D respectively) and enzyme activity (C and E respectively) in the TE model. Untreated controls are represented by the open bars and TE and T24-treated rats administered with vehicle, flutamide or FSH.Ab are represented by the closed bars. The housekeeping gene (A; INT, intensity of band) and testis weight (F) are also represented. *Significant difference ($P < 0.05$) compared with untreated control. Differences between treatment groups are represented at $P < 0.05$. Data are expressed as means $\pm$ S.E.M. ($n = 7$ or 8).

Figure 2 Testicular androgens in the TE model. Testicular concentration of testosterone (A; note log scale on y axis), DHT (B) and 3α-Adiol (C) in untreated control and TE-suppressed animals. Formation of 5α-reduced androgens as a percentage of testosterone ((DHT + 3α-Adiol/testosterone) × 100) represented in (D). *Significant difference ($P<0.05$) compared with untreated control. **Significant difference ($P<0.05$) compared with TE vehicle. Data expressed as means ± S.E.M. ($n=7$ or 8). Note log scale on y axis in (A) and (D).

Table 4 GnRH immunisation (GnRH_{imm}) model: serum LH and FSH, means ± S.E.M. (see Table 2 and Methods section for further details)

	LH (ng/ml)	FSH (ng/ml)
Treatment group		
Untreated control	0.74 ± 0.19	3.92 ± 0.23
GnRH _{imm} +vehicle	0.09 ± 0.01*	2.1 ± 0.08*
GnRH _{imm} +FSH.Ab	0.05 ± 0.01*†	NA
GnRH _{imm} +rhFSH	0.11 ± 0.01*	NA
GnRH _{imm} +hCG+vehicle	0.06 ± 0.01*	2.95 ± 0.08*†
GnRH _{imm} +hCG+FSH.Ab	0.06 ± 0.01*	NA

NA denotes not assayed due to interference of FSH.Ab or rhFSH in the FSH RIA. *Significant difference compared with untreated control. †Significant difference compared with GnRH_{imm}+vehicle.

testosterone) and this increase was not affected by FSH.Ab or rhFSH treatment (Fig. 4D). Administration of hCG to GnRH-immunised animals decreased the conversion of testosterone to 5α-reduced metabolites compared with GnRH-immunised+vehicle-treated animals.

Discussion

This study aimed to investigate the effects of testosterone and FSH on 5αR-1 and 5αR-2 mRNA and enzyme activity in adult rat testis in order to understand the hormonal regulation of the testicular 5αR isoforms and their relative importance in maintaining spermatogenesis. Two *in vivo* models of spermatogenic suppression were used: TE implants selectively suppressed LH/testosterone but not FSH, whereas GnRH immunisation suppressed both testosterone and FSH. This study showed that the testicular 5αR isoforms are differentially regulated by FSH and testosterone at the level of gene expression, and provides a better understanding of the control of testicular androgen biosynthesis.

Effect of testosterone on 5αR-1 and 5αR-2

When the TE suppression model was used to suppress testicular testosterone, 5αR-1 mRNA and enzyme activity increased, and this stimulatory effect was enhanced further by blocking residual androgen levels with the AR antagonist, flutamide. These data suggest that testosterone negatively regulates the expression of the 5αR-1 isoform. In support, restoration of testosterone by administration of hCG in combination with FSH.Ab (which neutralised the hCG-induced concomitant increase in FSH in this species) to GnRH-immunised animals decreased 5αR-1 mRNA and enzyme activity. Partial restoration of testosterone with T24 treatment, however, did not suppress 5αR-1 as did hCG, presumably because the concentration of testosterone was not increased sufficiently to inhibit 5αR-1. Evidence in support of this is seen from testicular steroid levels in these model systems. The amount of testosterone

in a normal adult rat was ~ 100 ng/g testis and this was suppressed to ~ 2 ng/g testis with GnRH immunisation. Whereas hCG treatment fully restored testosterone (~ 300 ng/g testis), T24 treatment only partially restored testicular testosterone (~ 12 ng/g testis; O'Donnell *et al.* 1999). Thus the suppression of 5 α R-1 mRNA and activity by testicular testosterone presumably requires levels greater than 12 ng/g testis.

Negative regulation of 5 α R-1 isoform by testosterone is consistent with our hypothesis that a reduction in testicular testosterone leads to an increase in the metabolism of testosterone to the more potent metabolite, DHT. Thus, when testicular testosterone levels are low and unable to maintain sperm production, amplification of the androgenic stimulus via conversion of testosterone to DHT would enable continued low levels of spermatogenesis. Negative regulation of 5 α R-1 by testosterone has been demonstrated in a number of tissues, such as liver (Yates *et al.* 1958, Lopez-Solache *et al.* 1996) and adrenal cortex (Lephart *et al.* 1991). Unlike the 5 α R-1 isoform, which was shown to be regulated by testosterone, the 5 α R-2 isoform in rat testis was not regulated by testosterone. Thus, neither suppression nor restoration of testosterone affected 5 α R-2 mRNA or enzyme activity. This is in contrast to the ventral prostate where 5 α R-2 is positively regulated by androgens (Andersson & Russell 1990). The reason for the differential regulation of the 5 α R isoforms in different tissues is unknown, but suggests that factors that regulate the 5 α R isoforms may be tissue-specific.

Effect of FSH on 5 α R-1 and 5 α R-2

Studies in the literature that have investigated the regulation of 5 α R-1 are controversial, and have measured only enzyme activity in immature rat testis. Some studies showed an effect of FSH on 5 α R-1 enzyme activity (Nayfey *et al.* 1975, Welsh & Wiebe 1976) whereas others did not (Dorrington & Fritz 1975, Muroso & Payne 1979). The present study showed that acute (6 day) FSH suppression, by administering FSH.Ab to TE-treated rats, decreased 5 α R-1 mRNA, and that FSH restoration, by rhFSH or hCG treatment to GnRH-immunised animals, increased 5 α R-1 mRNA. These findings demonstrate that FSH positively regulates the 5 α R-1 isoform in rat testis at the mRNA level.

Figure 3 5 α R-1 and 5 α R-2 mRNA (B and D respectively) and enzyme activity (C and E respectively) in the GnRH immunisation model. Untreated control animals are represented by the open bars and GnRH-immunised (GnRH_{imm}) and GnRH_{imm}+hCG-treated rats administered vehicle, FSH.Ab or rhFSH are represented by closed bars. The housekeeping gene (A) and testis weight (F) are also represented. *Significant difference ($P < 0.05$) compared with untreated control. **Significant difference ($P < 0.05$) compared with GnRH_{imm}+vehicle. Significant differences between treatment groups represented at $P < 0.05$. Data are expressed as means $\pm$ S.E.M. ($n = 8$).

Figure 4 Testicular androgen concentrations in the GnRH immunisation model. Testicular concentration of testosterone (A; note log scale on y axis), DHT (B) and 3 α -Adiol (C) in control and GnRH-immunised (GnRH_{imm}) animals. Formation of 5 α -reduced androgens as a percentage of testosterone ((DHT+3 α -Adiol/testosterone) $\times$ 100) represented in (D). *Significant difference ($P < 0.05$) compared with untreated control. **Significant difference ($P < 0.05$) compared with GnRH_{imm}+vehicle. Data are expressed as means $\pm$ S.E.M. ($n = 8$).

Whereas FSH positively regulated 5 α R-1 mRNA, neither suppression nor restoration of FSH changed 5 α R-1 enzyme activity, suggesting that the regulation of the

5 α R-1 isoform by FSH in these studies occurred at the gene expression level and did not affect the translation of 5 α R-1 mRNA into protein within the time-frame studied here. There are numerous reports in the literature that demonstrate that the level or change in 5 α R-1 mRNA does not necessarily follow 5 α R-1 protein levels. For example, in humans 5 α R-1 mRNA expression is clearly detectable whereas there are undetectable levels of 5 α R-1 protein (Jenkins *et al.* 1992, Thigpen *et al.* 1993b). Viger & Robaire (1995) showed that in the rat, 5 α R-1 mRNA levels at day 90 was about one-third of that of day 30, whereas 5 α R-1 enzyme activity at day 90 was $\sim$ 30-fold less than that at day 30 (Rivarola *et al.* 1972, Pratis *et al.* 2000). Thus, there appears to be post-transcriptional or translational modification of the 5 α R-1 isoform. In rat testis, the 5 α R-1 transcript has been demonstrated to change in size with age, such that a 2.5 kb 5 α R-1 transcript is expressed during immaturity when 5 α R-1 activity is high, and a 2.7 kb transcript is expressed in the adult, which coincides with the period when 5 α R-1 activity levels are low, suggesting that 5 α R-1 enzyme activity may depend on the transcript size of the 5 α R-1 mRNA (Viger & Robaire 1995).

There are several studies showing discordant patterns of mRNA and protein expression. For example, DHT decreased AR mRNA but increased AR protein in LnCaP and MDA453 cell lines (Yeap *et al.* 1999) and in rat prostatic smooth muscle cells, androgen withdrawal increased steroid sensitive gene 1 (SSG1) mRNA whereas SSG1 protein decreased (Marcantonio *et al.* 2001). The discordant response between mRNA and activity for this study (described above) could relate to differences in the half-life of the mRNA and protein. The half-life of recombinant 5 α R-1 protein is $\sim$ 30 h (Thigpen *et al.* 1993a) whereas the half-life of 5 α R-1 mRNA is unknown. A short half-life would indicate that mRNA and enzyme activity levels would be critically dependent on the time-point at which measurements were taken, suggesting that further studies are required to determine the changes in 5 α R-1 mRNA and activity at different time-points following treatment.

5 α R-2 enzyme activity was suppressed by GnRH immunisation, and restoration of FSH by rhFSH or hCG treatment following GnRH immunisation, increased 5 α R-2 enzyme activity to control levels. These findings suggest that FSH positively regulates 5 α R-2 enzyme activity. In support, blocking the concomitant rise in FSH that occurs with hCG treatment prevented the restoration of 5 α R-2 activity, providing further evidence that FSH positively regulates 5 α R-2 enzyme activity. Whereas FSH affected 5 α R-2 enzyme activity, FSH did not change the levels of 5 α R-2 mRNA. The FSH-induced increase in 5 α R-2 enzyme activity but not mRNA could be due to the time-point at which samples were collected after treatment, as described above for 5 α R-1. It is conceivable that FSH caused an initial increase in mRNA within

1–2 days of treatment, which then declined to control levels. The half-life of recombinant 5 α R-2 protein is similar to that of recombinant 5 α R-1 (~30 h; Thigpen *et al.* 1993a), whereas the half-life of 5 α R-2 mRNA is unknown. As for 5 α R-1, further studies are required to measure 5 α R-2 mRNA and activity over several time-points. Additional studies are also required to determine the effect of other hormones on the regulation of the 5 α R isoforms. For example, in genital skin fibroblasts, which exclusively express the 5 α R-2 isoform, 5 α R-2 activity has been shown to be regulated by DHT and insulin-like growth factor-I (Horton *et al.* 1993), transforming growth factor- β 1 and - β 2 (Wahe *et al.* 1993) and activin A (Antonipillai *et al.* 1995).

As mentioned above, 5 α R-2 enzyme activity decreased when FSH.*Ab* was co-administered with hCG. Surprisingly, co-administration of FSH.*Ab* with hCG increased 5 α R-2 at the mRNA level. However, in the TE suppression model, FSH.*Ab* administration decreased 5 α R-2 mRNA. The discrepancy between these findings is unexplained but it is possible that the increase in 5 α R-2 mRNA after FSH suppression in hCG-treated animals is partly due to the presence of testosterone in these animals. A difference between these *in vivo* models is that GnRH immunisation produces more severe spermatogenic regression and FSH is chronically suppressed in GnRH-immunised animals, whereas FSH is only acutely suppressed in the TE-suppression model.

5 α R isoform mRNA and activity were expressed on a per gram of testis basis in order to investigate the testicular concentrations of these enzymes, and their relationship to the testicular androgen concentration. The significant changes in 5 α R-1 and 5 α R-2 induced by hormonal treatment of TE-treated or GnRH-immunised rats, in the absence of changes in testis weight, may be a consequence of modulation of the regulation and expression of these enzymes in Leydig and Sertoli cells, both of which have been demonstrated to contain at least 5 α R-1 (Dorrington & Fritz 1975, Nayfey *et al.* 1975, Welsh & Wiebe 1976, Murono & Payne 1979, Viger & Robaire 1995). Since restoration of testosterone and/or FSH in these models cause increases in some germ cell populations (Meachem *et al.* 1998), it is also possible that the changes in the testicular concentration of 5 α R isoforms is related to the appearance of certain germ cell types. Although it seems that 5 α R-1 is present predominantly in Leydig cells (Viger & Robaire 1995) there is also evidence for 5 α R activity (probably 5 α R-1) in seminiferous tubules, in Sertoli cells as well as germ cells (Dorrington & Fritz 1975). The localisation of 5 α R-2 in the testis is unknown.

Interpretation of changes in the concentration of 5 α R isoforms between untreated controls and treated (TE-treated or GnRH-immunised) animals is complicated by the fact that there are large changes in testis weight induced by these treatments. These differences in testis weight are related to the disappearance of various germ

cells from the testis (Meachem *et al.* 1998) and are not likely to be related to changes in Sertoli cell numbers (McLachlan *et al.* 1995). The data expressed per gram of testis show that TE-treated and GnRH-immunised animals have an increase in the concentration of 5 α R-1 activity; however, if the data were expressed on a per testis basis no changes would be seen. Nevertheless, analysis of the testicular androgen levels showed that the ability of the testis to produce 5 α -reduced androgens is increased when the testis is in a hypogonadotrophic state. This is demonstrated by an increase in the amount of DHT and 3 α -Adiol produced when taking into account the large decrease in substrate (testosterone) concentration. Thus, TE treatment or GnRH immunisation increased the capacity of the testis to produce 5 α -reduced androgens. This increased capacity correlated with an increase in the testicular concentration of 5 α R-1 activity, suggesting that the concentration of this enzyme in particular is important for the production of 5 α -reduced androgens in the testis.

Thus, this study showed that the concentration of 5 α -reduced metabolites in the testis is influenced by expression of 5 α R-1 rather than 5 α R-2, and that the 5 α R-1 isoform appears to be negatively regulated by testosterone. Thus 5 α R-1, which is the predominant 5 α R isoform in rat testis (Pratis *et al.* 2000), is important for testicular 5 α -reduced androgen biosynthesis. We have shown previously in rats that synthesis of 5 α -reduced androgens in the testis is particularly important for the maintenance of androgenic effects on spermatogenesis when testicular testosterone levels are experimentally lowered in a model of contraception (O'Donnell *et al.* 1996, 1999). The results presented here extend the previous studies to suggest that when testicular testosterone levels are low, the concentration of 5 α R-1 in the testis is increased, thereby allowing increased production of the more potent androgen DHT, and hence amplification of the androgenic stimulus on spermatogenesis. In terms of relevance to clinical contraceptive studies, the maintenance of testicular 5 α -reduced androgen levels during testosterone-based contraception has been suggested to be associated with the continued low level of sperm production in oligospermic responders (Anderson *et al.* 1996, 1997). Therefore we speculate that increases in testicular 5 α R-1 activity in the human testis may occur during contraceptive treatment, as a consequence of the negative regulation of 5 α R-1 expression by testosterone, and may underlie the maintenance of spermatogenesis in oligospermic men. Further studies are required to assess 5 α R-1 expression and activity in the human testis. Considering that 5 α R-1 appears to be the predominant 5 α R expressed in human testis (Aumuller *et al.* 1996) and that administration of specific 5 α R-2 inhibitors failed to induce complete suppression of spermatogenesis in men undergoing hormonal contraception (McLachlan *et al.* 2000, Kinniburgh *et al.* 2001), it is possible that selective inhibition of 5 α R-1 or dual inhibition of both 5 α R

isoforms may be warranted to improve the degree of spermatogenic suppression during testosterone-based contraceptive administration. In addition, our studies show that FSH can influence 5 α R-1 and 5 α R-2, suggesting that circulating levels of FSH could also modulate 5 α -reduced androgen biosynthesis in the testis. In summary, this study suggests that the concentrations of testicular 5 α R isoforms are hormonally regulated, and highlights the contribution of the 5 α R-1 isoform to 5 α -reduced androgen biosynthesis.

Acknowledgements

This study was supported by the National Health and Medical Research Council of Australia Program Grant (No. 983212). L O'D received support from the Wellcome Trust Research Training Fellowship in Reproductive Biology (No. 050387). We would like to thank Fiona McLean for the analysis of testicular steroids.

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Received 26 September 2002

Accepted 26 November 2002