Role of FSH, numbers of FSH receptors and testosterone in the regulation of inhibin secretion during the seasonal testicular cycle of adult rams

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The regulation of inhibin secretion has not been elucidated fully in male ruminants. The aim of this study was to determine the relative importance of FSH and testosterone concentrations, and FSH receptors, in the control of secretion of immunoactive inhibin in rams. In Expt 1, temporal changes in hormone concentrations and testicular FSH binding were determined for two groups of rams ($n = 4$) kept under opposite, alternating 4 month periods of long (16 h light:8 h dark) and short (8 h light:16 h dark) days. Testicular biopsies (1–2 g) were collected when the testes were regressed, redeveloping, redeveloped and regressing. In Expt 2, separate groups of rams ($n = 4$) kept under natural photoperiod (latitude 45°48' N) were designated as controls or passively immunized (for 3 weeks) with sufficient oestradiol antiserum to increase testosterone secretion without altering LH and FSH; this was done when the testes were regressed (non-breeding season) and redeveloped (breeding season). In both groups of rams (Expt 1), 'seasonal' increases in FSH concentrations began a few weeks earlier than did increases in inhibin concentrations. FSH reached maximum concentrations during testicular recrudescence, whereas numbers of FSH

receptors in the testis and circulatory inhibin concentrations did not reach peak values until the testes were fully developed. Numbers of FSH receptors per testis, but not FSH concentration, were positively correlated ($r = 0.65$) with inhibin concentrations across the four stages of the testicular cycle. Near the end of testicular recrudescence early in the breeding season (Expt 2), relatively high FSH concentration was associated with increased abundance of FSH receptor mRNA (90%) and number of receptors (45%) in the testis and increased inhibin concentrations (50%), compared with when the testes were regressed. Moderate, physiological increases in testosterone secretion in immunized rams did not affect inhibin in either season. These results indicate that: (i) FSH stimulation of immunoactive inhibin secretion by Sertoli cells as testes recrudescence is via increases in secretion (early) and cognate receptors (late); (ii) FSH upregulates the synthesis of its own receptor late in recrudescence; and (iii) the positive correlation ($r = 0.70$) observed between circulatory testosterone and immunoactive inhibin does not reflect a causal relationship.

Introduction

Inhibin is a heterodimeric glycoprotein named for its regulatory effect on FSH synthesis and secretion. It consists of an α -subunit and one of two related β -subunits (β_A and

β_B) (Gnessi *et al.*, 1997). A dimeric ovine inhibin of about 32 kDa has been isolated from rete testis fluid of adult rams and the N-terminal sequence of its subunits has been characterized (Vale *et al.*, 1988; Bardin *et al.*, 1989). The inhibin A ($\alpha\beta_A$) form was identified, but not inhibin B ($\alpha\beta_B$), indicating that inhibin A may be the predominant dimer produced in the testis and present in peripheral blood of rams. In male primates (Illingworth *et al.*, 1996; Plant *et al.*, 1997) and rodents (Woodruff *et al.*, 1996; Abel *et al.*, 2000; Jin *et al.*, 2001), inhibin B is known or believed to be the primary dimeric form in the blood circulation. Inhibin concentration in rams is increased in late fetal life (Phillips *et al.*, 1992; Miller *et al.*, 1997) and in the first few months after birth (Schanbacher, 1988; Sanford *et al.*, 2000) when

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Sertoli cells are completing proliferation (Hochereau-de Reviers *et al.*, 1987). A comparatively small increase in inhibin occurs in mature rams during seasonal testicular recrudescence (Sanford *et al.*, 2000). Inhibin is involved in suppression of FSH secretion in late fetal life (Miller *et al.*, 1997), during puberty (Al-Obaidi *et al.*, 1987; Tilbrook *et al.*, 1999a) and in adulthood (McKeown *et al.*, 1997; Tilbrook *et al.*, 1999b). Furthermore, dimeric inhibin and forms of the α -subunit of inhibin may act as paracrine/autocrine regulators in the testis (Schneyer *et al.*, 1991; Gnessi *et al.*, 1997; Chong *et al.*, 2000).

Most of the inhibin in the blood pool comes from the gonad (Schanbacher, 1988; Miller *et al.*, 1997; Tilbrook *et al.*, 1999a) and the Sertoli cells in particular. Sertoli cells in the testes of ram fetuses appear to express all three inhibin subunits (α , β_A and β_B) (Thomas *et al.*, 1995; Jarred *et al.*, 1999), as do Leydig cells until day 90 of fetal development when the α -subunit is no longer detectable (Jarred *et al.*, 1999). This is consistent with the finding that Leydig cells in adult rams do not secrete inhibin in response to hCG or endogenous LH (Tilbrook *et al.*, 1991). FSH is a primary regulator of inhibin secretion by Sertoli cells, stimulating synthesis of the α -subunit, which is produced in excess to the β -subunit (Bardin *et al.*, 1989; Steinberger *et al.*, 1992). In rams, FSH administration increases inhibin production by fetal testis (Albers *et al.*, 1989) and the output of inhibin into the testicular lymphatic and vascular systems in adults (Voglmayr *et al.*, 1992). Corresponding increases in FSH, immunoactive inhibin (Lincoln and McNeilly, 1989; Sanford *et al.*, 1993) and inhibin A (Lincoln *et al.*, 2001) during testicular redevelopment, and greater numbers of FSH receptors in the testis in the breeding season compared with the non-breeding season (Barenton and Pelletier, 1983; Monet-Kuntz *et al.*, 1988), provide additional evidence for FSH regulation of inhibin secretion in rams. However, the mode of FSH action (altered secretion versus number of receptors) and the regulation of gene expression for FSH receptors as testes pass through the different stages of the seasonal cycle are poorly understood. In the present study, experiments were designed to clarify the temporal relationship between FSH and inhibin concentrations, and the number of FSH receptors across specific stages of the testicular cycle. In particular, the hypotheses that changes in FSH receptors as well as FSH concentrations are responsible for changes in inhibin secretion, and that FSH modulates the synthesis of its cognate receptor, were tested.

Inhibin secretion by Sertoli cells may also be regulated by paracrine mechanisms acting within the testis. Sertoli cells in rodent species and human males have androgen receptors, and normal cell function requires both testosterone and FSH (Sar *et al.*, 1993). Thus, testosterone from neighbouring Leydig cells is a candidate for inhibin regulation. An effect of testosterone may also be mediated indirectly by other androgen target cells such as peritubular myoid cells. Rat peritubular cells secrete a non-mitogenic paracrine factor, PmodS, in response to testosterone, which stimulates the secretion of a number of Sertoli cell proteins,

including inhibin (Gnessi *et al.*, 1997). Other studies, mainly with Sertoli cells in culture, give conflicting indications as to whether testosterone stimulates or inhibits inhibin secretion (Bardin *et al.*, 1989; Risbridger and de Kretser, 1989). Sertoli cells and peritubular cells in ram testis contain androgen receptors (Lincoln *et al.*, 1996), and seasonal variations in blood inhibin and testosterone concentrations are positively correlated (Lincoln and McNeilly, 1989; Sanford *et al.*, 1993). The possibility that there is a causal relationship between these two hormones has not been examined. This aspect of paracrine regulation of inhibin secretion was investigated in the present study in a novel experimental model using oestradiol immuno-neutralization. In this model, testosterone secretion in rams is increased without increasing LH and FSH stimulation to the testes (Sanford, 1987, 1989; Sanford *et al.*, 1991). The hypothesis that this increased testosterone production would alter inhibin secretion from the testis was tested.

Materials and Methods

Animals

Adult DLS (Dorset–Leicester–Suffolk) rams ($n = 24$) were used in this investigation. Rams of the relatively new DLS breed of sheep show moderate changes in most of their reproductive endocrine traits during the seasonal testicular cycle (Sanford and Robaire, 1990; Sanford *et al.*, 1993). Animals were provided from the Agriculture and Agri-Food Canada research flock in La Pocatière, Québec. They were fed a diet of hay and grain, and had free access to a mineral–vitamin mixture and drinking water. Animal experimentation was done in accordance with guidelines of the Canadian Council on Animal Care and the Animal Care Committee of McGill University.

Experimental protocols

In Expt 1, four rams were placed in each of two adjacent photoperiod-controlled rooms measuring 3 m $\times$ 3 m. The rams were subjected to alternating 4 month periods of stimulating (8 h light:6 h dark) and inhibiting (16 h light:8 h dark) daylengths, beginning with either short days (group 1 rams) or long days (group 2 rams). Light intensity in the centre of the rooms at the head level of the rams was 90 lux. After the testicular cycle of rams was entrained to the prevailing photoperiods in the initial 12 months, scrotal circumference and blood hormone concentrations (testosterone, inhibin and FSH) were determined at frequent intervals for the next 14 months. Rams were 30-months-old and weighed 74.6 ± 3.0 kg at the onset of the experimental period, which was equivalent to nearly two 'seasonal' testicular cycles. Body weight of the rams increased ($P < 0.01$) by 15% during the first 7 months, but remained stable thereafter when testicular biopsies were taken in regressed, redeveloping, redeveloped and regressing stages of the testicular cycle to assess FSH receptor binding activity.

In Expt 2, separate groups of four rams (aged 6 years) kept

indoors, but exposed to natural changes in photoperiod (latitude 45°48' N), were passively immunized against oestradiol for 3 weeks beginning in mid-August or mid-March. Other groups of four rams served as controls. Blood samples were collected for hormonal analyses (testosterone, inhibin and FSH) on day 21 of immunization and, on day 23, rams were gonadectomized and testicular tissue was collected to assess FSH receptor binding activity and mRNA abundance. Collections were made when testicular size and testosterone secretion in adult DLS rams are normally near or at yearly high and yearly low values (Sanford and Robaire, 1990; Paquette, 1993), and when adult DLS ewes are showing oestrous activity or becoming seasonally anoestrous (Fahmy and Shrestha, 1992). Body weight of rams, recorded at the time of gonadectomy, was 20% higher ($P < 0.01$) in the breeding season (90.9 ± 2.4 kg) than in the non-breeding season (75.7 ± 3.5 kg). The testosterone results presented in this study have been reported elsewhere (Price *et al.*, 2000).

Passive immunization against oestradiol

The antiserum used in passive immunization (Expt 2) was raised in gonadectomized, adult DLS rams against oestradiol-6-(0-carboxymethyl)oxime-BSA. Active immunization procedures and preparation of antisera harvested for use in the present study are described elsewhere (Sanford, 1987; Squire *et al.*, 1993). The pool of antiserum prepared had a titre of 1:4500 and was specific for oestradiol; it crossreacted only slightly with oestrone (15%). Passively immunized rams were injected (jugular vein) with 60 ml oestradiol antiserum every 3 or 4 days for 3 weeks (total of seven injections). In the breeding season, the amount was increased to 120 ml in week 2 to take into account the 50–100% higher oestradiol concentration in peripheral blood of DLS rams when testes are in the redeveloped state (Sanford *et al.*, 1993). This immunization protocol was used to produce an antibody titre in rams of about 1:200 within a week or so, and an LH-independent increase in testosterone secretion by week 3 (Sanford, 1987, 1989; Sanford *et al.*, 1991).

Blood sampling and hormone assays

Blood samples were collected serially from rams on 17 occasions during the 14 month experimental period in Expt 1 and on one occasion at the end of the immunization period in Expt 2. A sample of blood (7 ml) was withdrawn from the jugular vein by venepuncture every 20 min for either 6 h (Expt 1) or 8 h (Expt 2), beginning at about 09:30 h. Mean hormone concentrations were determined by assaying serum pools (equal aliquots of 18 or 25 sera) representing individual rams and sampling periods, with one exception. In Expt 2, mean and basal (interpulse) testosterone concentrations were determined by averaging values of all individual sera or only the lowest values not associated with testosterone pulses, defined as described by Sanford *et al.* (1993).

Double-antibody radioimmunoassay procedures were used to determine serum testosterone (Sanford, 1985) and FSH (Cheng *et al.*, 1981) concentrations. The reference preparation in the FSH assay was NIH-FSH-S12. Minimum detectable concentrations of testosterone and FSH at the serum volumes indicated were 0.20 ng ml^{-1} (50 μl) and 2.2 ng ml^{-1} (100 μl), respectively. Immunoactive inhibin concentration was measured using the double-antibody radioimmunoassay procedure developed by Vaughan *et al.* (1989). The primary antiserum was raised in a rabbit against a synthetic peptide fragment of porcine inhibin α -subunit (1–26 AA sequence of the N-terminus). The assay used human inhibin α 1–25 subunit as tracer and ovine inhibin α 1–25 subunit as the reference preparation. The primary antibody detects forms of the free α -subunit of inhibin, as well as dimeric inhibin; however, measures of inhibin using antisera generated against the synthetic porcine inhibin α -subunit fragment increase in parallel to specific measures of dimeric inhibin A during testicular recrudescence (Lincoln and McNeilly, 1989; Lincoln *et al.*, 2001). Unknown values have been expressed as pmol 32 kDa inhibin ml^{-1} . Minimum detectable concentration was $0.30 \text{ pmol ml}^{-1}$ (50 μl). Intra- and interassay coefficients of variation in these procedures were all $< 12\%$.

Collection of testicular tissue

Testicular biopsies were taken from rams (Expt 1) as described by Sanford (1989). Between 1 g and 2 g of tissue was excised after a 2–3 cm incision was made into the mid-region of the scrotal sac and underlying layers of the testicular capsule. Tissue samples were frozen in liquid nitrogen and stored at -70°C until analysed for FSH receptor binding activity. Gonadectomy of rams (Expt 2) was as reported by Price *et al.* (2000). After the testes were removed from rams and weighed, 1–2 g samples of testicular tissue were taken to determine FSH receptor binding activity and mRNA abundance. Tissue for RNA extraction was placed immediately into 4 mol guanidinium isothiocyanate l^{-1} containing 0.12 mol β -mercaptoethanol l^{-1} . All samples were frozen on dry ice and stored at -70°C .

FSH receptor binding assay

Established procedures (Yarney *et al.*, 1988) were used to determine FSH receptor binding activity in the testis. Methods for membrane extraction, radioiodination of FSH and assay of FSH receptors were modified slightly in Expt 2. Pieces of testicular tissue were homogenized in cold 25 mmol Tris-HCl (pH 7.2) l^{-1} buffer containing 250 mmol sucrose l^{-1} , 100 μg bacitracin ml^{-1} (Sigma, St Louis, MO) and 1 mmol phenylmethane sulphonyl fluoride (PMSF) l^{-1} (Sigma). Tissue homogenates were centrifuged at 500 g for 30 min, followed by centrifugation of supernatant at 30 000 g for 30 min to recover membrane fractions. The membrane pellets were washed and resuspended in 25 mmol Tris-HCl l^{-1} buffer (pH 7.2) containing 0.2% (w/v) MgCl_2 and PMSF. Membranes were snap frozen in liquid

nitrogen and stored at -70°C until assayed. For the assay, highly purified human FSH (Sairam *et al.*, 1978) was labelled with ^{125}I (IodoGen; Pierce Chemical Co., Rockford, IL). Radiolabelled FSH was purified on Bio-Rad Bio-Gel P-6 gel desalting columns (Bio-Rad, Richmond, CA). Single point binding assays were carried out in duplicate in assay buffer (25 mmol Tris-HCl l^{-1} , pH 7.2, 0.2% (w/v) MgCl_2 , 0.1% (w/v) BSA) with near saturating concentration of radiolabelled hormone (5 ng). In both experiments, the specific activity of labelled FSH ranged from 54 to 75 μCi ($\mu\text{g hormone}^{-1}$) (1 Ci = 37 GBq). The biologically active fraction, determined by the percentage binding of labelled FSH to excess pig or sheep testicular membranes, was 28–39%. Non-specific binding in the presence of 1 μg unlabelled ovine FSH was < 20% of total binding. Intra- and interassay coefficients of variation were < 12%.

Membrane protein content was determined by the Bio-Rad assay (Bio-Rad) in Expt 1 and by the bicinchoninic acid method (Smith *et al.*, 1985) in Expt 2, with reagents supplied by Pierce Chemical Co. Protein estimates were 30% lower using the latter method. Aliquots of membrane fractions were first solubilized by boiling for 30 min in 0.5 or 1.0 mol NaOH l^{-1} and assayed after neutralization with HCl. BSA was used as a standard.

The amount of membrane-bound FSH, taken to reflect the number of unoccupied FSH receptors, was determined in the equivalent of 25 mg wet tissue. Binding activity was assessed on a per mg membrane protein, per g tissue and per testis basis.

FSH receptor mRNA analysis

Testicular tissue (Expt 2) was thawed and homogenized in 4 mol guanidium isothiocyanate l^{-1} containing 0.12 mol β -mercaptoethanol l^{-1} . Total RNA was isolated by centrifugation for 21 h at 174 000 g on a cushion of 5.7 mol caesium chloride l^{-1} . The pellet was resuspended in 3 mol sodium acetate l^{-1} , precipitated twice with ethanol and quantified by measuring absorbance at 260 nm.

Total RNA was analysed by slot blot hybridization (6 μg) as described by Sambrook *et al.* (1989) and membranes were cross-linked (150 mJ) in a commercial UV chamber (Bio-Rad, Mississauga, ON). The membranes were incubated for 2 h in prehybridization solution containing 10% (w/v) dextran sulphate, 5-strength saline-sodium phosphate-EDTA (SPPE), 5-strength Denhardt's solution, 0.5% (w/v) sodium dodecyl sulphate (SDS) and herring sperm DNA (200 mg ml^{-1}). Hybridization with a labelled bovine cDNA probe was performed at 65°C overnight. After hybridization, membranes were washed twice in double strength SSPE-0.1% (w/v) SDS at room temperature and twice at 65°C . The labelled membranes were exposed to Kodak X-Omat film (Kodak Canada Inc., Toronto, ON) at -70°C in the presence of an intensifying screen for 1–5 days. The membranes were stripped and rehybridized to a labelled 28S ribosomal cDNA probe as an internal control. Relative abundance of signal was quantified by densitometry

(Collage Analysis; Fotodyne Inc., Hartland, WI). The density of hybridization signals was corrected for loading using hybridization to 28S and these corrected data were expressed relative to a control ram sample that was included in the two blots.

The bovine FSH receptor cDNA probe was a 1264 bp fragment generated by RT-PCR, representing a portion of the extracellular binding domain to the 3' stop codon (Houde *et al.*, 1994). The probe was labelled with [α ^{32}P]-dCTP by random primer extension (T7 Quickprime; Pharmacia, Dorval, QC) and was purified by centrifugation through a minicolumn using Wizard PCR Prep DNA purification (Promega, Ottawa, ON).

Data analyses

In Expt 1, scrotal circumference was recorded approximately every 2 weeks during the 8 month 'seasonal' testicular cycles. Only values corresponding closely to the days of blood collection are presented. Paired-testes mass at the time of testicular biopsy was estimated from measurement of scrotal circumference using a regression equation reported by Knight (1977) for Romney rams ($r = 0.93$, $n = 25$) with similar measurements (range 27–36 cm). Weight estimates were used to determine relative differences in FSH receptor binding on a per gram of tissue and per testis basis between stages of the testicular cycle. Mean hormone concentrations at the time of biopsy were derived by extrapolation from concentrations determined for the period of blood sampling just before and afterwards.

All data from Expt 1 were subjected to ANOVA for repeated measures (SAS, 1989). The significance of month (within group) was determined by one-way ANOVA and of group and stage of testis cycle by two-way ANOVA. Two-way ANOVA was also used to determine differences in maximum values for scrotal circumference and hormone concentrations between groups and 8 month 'seasonal' cycles. When the main effect of time (month, stage, cycle) or the group $\times$ time interaction was significant ($P < 0.05$), comparisons of least-squares means were made using the PDIFF (probability difference) test. Where appropriate, a logarithmic transformation was applied in these analyses to normalize variances. Linear regression analysis was used to determine relationships between testicular size or serum concentration of immunoactive inhibin and selected endocrine variables. Pearson correlation coefficients were calculated for each group, either by pooling r values obtained on individual rams across months of their 'seasonal' cycles or on combined data for the four rams across stages of their testicular cycles. Correlations on individual rams were tested for homogeneity before pooling (Steel and Torrie, 1960). Non-homogeneity appeared only once (group 1, inhibin and previous FSH) and in this instance, the pooled r value represents three rather than four animals.

In Expt 2, two-way ANOVA was used to determine the significance of season and immunization (SAS, 1989).

Fig. 1. (a,b) Scrotal circumference and mean serum concentrations of (c,d) testosterone, (e,f) immunoactive inhibin and (g,h) FSH for group 1 (a,c,e,g) and group 2 (b,d,f,h) rams during alternating 4 month periods of stimulative (8 h light:16 h dark, ■) and inhibitive (16 h light:8 h dark, □) daylength. Values are mean $\pm$ SEM ($n = 4$ rams). Arrows (a,b) indicate times of testicular biopsy when testes were regressed, redeveloping, redeveloped and regressing.

When main effects or the season $\times$ immunization interaction were significant ($P < 0.05$), least-squares means were compared by the PDIF test. If necessary, data were first transformed to logarithms. Pearson correlation coefficients were calculated for select pairs of endocrine variables using data on all four groups of rams. One of the control rams (non-breeding season) had such an abnormally high LH release (Price *et al.*, 2000) and, to a lesser extent, FSH secretion, that the data for this ram were excluded from these analyses.

Results

Experiment 1

Scrotal circumference of rams increased ($P < 0.01$) by about 6 cm between mid-long days and mid-short days to maximal values that were similar between groups and 'seasonal' testicular cycles (Fig. 1a,b). Mean testosterone concentration increased similarly in rams of both groups during testicular recrudescence; highest concentrations in mid-short days were several-fold greater ($P < 0.01$) than the

Table 1. Correlations between scrotal circumference or serum immunoactive inhibin concentration and selected endocrine variables for group 1 and group 2 rams across the months of their 8 month 'seasonal' testicular cycles

	Scrotal circumference			Inhibin		
	Inhibin	FSH ¹	FSH ²	Testosterone	FSH ¹	FSH ²
Group 1	0.73**	0.26*	0.46**	0.69**	0.30*	0.71**
Group 2	0.72**	0.32*	0.65**	0.65**	0.42**	0.64**

FSH¹: corresponding mean concentration; FSH²: mean concentration about 4 weeks earlier.

* $P < 0.05$ and ** $P < 0.01$ with 52 (FSH²) or 60 (other variables) error df, except for group 1 inhibin and FSH² with 39 error df.

lowest concentrations early in long days (Fig. 1c,d). Fluctuations in mean inhibin concentration were fairly moderate with highest concentrations in mid-short days only 75% (group 1, Fig. 1e) or 30% (group 2, Fig. 1f) higher ($P < 0.01$) than the lowest concentrations in the first or second month of long days. Inhibin peaks were lower ($P < 0.05$) in group 2 rams than in group 1 rams during both 'seasonal' cycles. Maximal mean FSH concentrations occurred relatively early in short days and were several-fold higher ($P < 0.05$) than minimal concentrations early in long days (Fig. 1g,h). There were no differences in peak FSH concentrations between groups or 'seasonal' cycles. For both groups, variations in inhibin concentration during the 14 month experimental period were related to variations in scrotal circumference and testosterone concentration ($r = 0.65$ – 0.73 , $P < 0.01$) (Table 1). Inhibin concentration and scrotal circumference changed in relation to FSH, more noticeably with concentrations about 4 weeks earlier ($r = 0.46$ – 0.71 , $P < 0.01$) than with those concurrent ($r = 0.26$ – 0.42 , $P < 0.05$).

The first in a series of four testicular biopsies was taken early in month 7 when testes were either regressed under long days (group 1, Fig. 1a) or redeveloped under short days (group 2, Fig. 1b). Subsequent biopsies taken 2 months apart clearly coincided with the remaining three stages of the testicular cycle in each group. Irrespective of when in the testicular cycle biopsy began, scrotal circumference was similar for rams of both groups in each stage, averaging 30.3 ± 0.7 cm ($n = 8$) in the regressed stage and 35.4 ± 0.9 cm in the redeveloped stage. Larger scrotal circumference measurements with testicular redevelopment corresponded to an increase ($P < 0.01$) in estimated testicular mass of 40% (Fig. 2a). Mean testosterone concentration was threefold higher ($P < 0.01$) when testes were redeveloped than regressed. Unlike testicular mass, testosterone concentration decreased to regressed rather than intermediate values in the regressing stage (Fig. 2b).

In group 1 rams, mean inhibin concentration was successively higher ($P < 0.05$) in the redeveloping (25%) and redeveloped (70%) stages of the testicular cycle compared with the regressed stage (Fig. 2c). Corresponding increases in inhibin in group 2 rams were considerably

Fig. 2. Estimated (a) testicular mass, mean serum concentrations of (b) testosterone, (c) immunoactive inhibin and (d) FSH, and (e) FSH receptor (FSHr) binding per testis for group 1 (□) and group 2 (■) rams during the regressed, redeveloping, redeveloped and regressing stages of their 'seasonal' testicular cycles. Values are mean $\pm$ SEM ($n = 4$ rams). Within groups, bars with different letters (a–c or x–z) are significantly different ($P < 0.05$). Asterisks indicate significant (* $P < 0.05$ or ** $P < 0.01$) differences between groups.

lower such that mean concentration in the developed stage was 40% lower ($P < 0.01$) than that for group 1 rams. Mean FSH concentration was highest in both groups in the

Table 2. Correlations (r) between estimated testicular mass or serum immunoactive inhibin concentration and selected endocrine variables for group 1 and group 2 rams across the four stages of their 'seasonal' testicular cycles

	Testicular mass			Inhibin		
	Inhibin	Mean FSH concentration	FSH receptor binding per testis	Testosterone	Mean FSH concentration	FSH receptor binding per testis
Group 1	0.73**	-0.23	0.74**	0.69**	0.15	0.71**
Group 2	0.56*	-0.42	0.69**	0.70**	-0.03	0.54*

* $P < 0.05$ and ** $P < 0.01$ with 14 error df.

Fig. 3. Mean serum concentrations of (a) immunoactive inhibin, (c) testosterone and (d) FSH, (b) basal (interpulse) testosterone concentration, (e) testicular FSH receptor (FSHr) mRNA abundance and (f) FSH receptor binding per testis for control (□) and immunized rams (■) in the non-breeding (early April) and breeding (early September) seasons. Hormonal and testicular measurements were performed on days 21 and 23, respectively, of passive immunization of treated rams against oestradiol. Estimate of mRNA abundance was corrected for loading (pixel density of target probe divided by pixel density of 28S rRNA probe) and is expressed relative to a control sample included in the blot. Values are mean $\pm$ SEM ($n = 3$ (controls, nonbreeding season) or $n = 4$ rams). ^{a-d}Bars with different letters are significantly different ($P < 0.05$).

redeveloping stage (Fig. 2d), and either decreased ($P < 0.05$) to regressed values in the regressing stage (group 1) or to lower than regressed values in the redeveloped and regressing stages (group 2). FSH concentration was different ($P < 0.05$) between groups in these last two stages. When expressed on a per mg protein or per g tissue basis, FSH receptor binding activity in the testis did not differ significantly between groups or stages of the testicular cycle. However, values in the redeveloped stage did tend to be higher (approximately 20%) than in the regressed stage, averaging 56.9 ± 4.1 fmol mg protein⁻¹ and 378 ± 25 fmol g tissue⁻¹ ($n = 8$). On a per testis basis, FSH receptor binding was estimated to be 70% higher ($P < 0.05$) in the redeveloped stage than in the regressed stage (Fig. 2e). FSH binding either remained high (group 2) or was decreased slightly (group 1) in the regressing stage. Considering all four stages of the testicular cycle, inhibin concentration and testis size

were correlated with FSH receptor binding per testis ($r = 0.54$ – 0.74 , $P < 0.05$) in both groups of rams (Table 2). Inhibin concentration was also related to testosterone concentration and testis size ($r = 0.56$ – 0.73 , $P < 0.05$).

Experiment 2

Passively immunizing rams against oestradiol for 3 weeks resulted in 75–100% increases ($P < 0.05$) in basal testosterone concentration in both the non-breeding and breeding seasons (Fig. 3b), and in mean testosterone concentration in the breeding season (Fig. 3c). Mean inhibin concentration was not influenced by immunization in either season (Fig. 3a), but was 50% higher ($P < 0.01$) in rams in the breeding season; this increase was associated with a fivefold higher ($P < 0.01$) mean testosterone concentration (control rams). The seasonal increase in inhibin also

Fig. 4. Correlation between (a) serum FSH concentration and testicular FSH receptor (FSHr) mRNA abundance, (b) testicular FSH receptor mRNA abundance and FSH receptor binding per testis and (c) FSH receptor binding per testis and serum immunoactive inhibin concentration. (a) $r = 0.62$, $P < 0.05$; (b) $r = 0.47$, $P < 0.08$; and (c) $r = 0.68$, $P < 0.01$. r values and probability values represent rams in both seasons ($n = 15$). Note the logarithmic scale for FSH concentration on the x axis in (a).

corresponded to a near sixfold increase ($P < 0.01$) in mean FSH concentration (Fig. 3d) and a 45% increase ($P < 0.01$) in FSH receptor binding in the testis (Fig. 3f). Increased FSH binding per testis was mainly the result of a 30% increase ($P < 0.05$) in testicular mass from 293 ± 22 g (non-breeding season, $n = 7$) to 377 ± 19 g (breeding season, $n = 8$). FSH receptor binding activity expressed on a per mg protein or per g tissue basis was not significantly higher in the breeding season (83.6 ± 5.8 fmol mg protein⁻¹, 418 ± 30 fmol g tissue⁻¹) than in the non-breeding season. The steady state mRNA abundance for FSH receptors was 90% higher ($P < 0.01$) in the breeding season (Fig. 3e) and, as for blood FSH concentration and FSH binding in the testis, was not affected by immunization.

Taking rams in both seasons into account, FSH concen-

tration (log₁₀) was related ($r = 0.62$, $P < 0.05$) to mRNA abundance for FSH receptors in the testis (Fig. 4a). A weaker relationship ($r = 0.47$, $P < 0.08$) was observed between abundance of FSH receptor mRNA and FSH binding per testis (Fig. 4b). FSH receptor binding per testis was correlated to a greater extent ($r = 0.68$, $P < 0.01$) with inhibin concentration (Fig. 4c).

Discussion

The regulation of inhibin secretion in seasonally breeding rams is not well understood. In particular, the relative roles of blood FSH and testosterone concentrations, and the number of FSH receptors in the testis, are unknown. In the present study, novel experiments are described which indicate that changes in FSH secretion and in numbers of FSH receptors *per se* regulate secretion of immunoactive inhibin during the seasonal testicular cycle of adult DLS rams, and that FSH induces gene expression for its own functional, membrane-bound receptor late in recrudescence. The stronger FSH signals provided to Sertoli cells, first by increases in FSH concentration and then by increased FSH receptors, are critical regulators of inhibin given the sequential nature of the relationship between FSH and inhibin secretion during the testicular cycle. Furthermore, using a unique *in vivo* model that permits selective increases in testosterone concentration, the results of the present study provide evidence that testosterone is not a major determinant of secretion of immunoactive inhibin in rams.

Inhibin concentration in peripheral blood of rams typically increased during testicular recrudescence (Expt 1). As adult testes become fully active, Sertoli cells do not increase in number, but their capacity for protein secretion (for example, androgen binding protein and inhibin) and for supporting increased numbers of germ cells as spermatogenesis becomes more efficient, does increase (Hochereau-de Reviers *et al.*, 1987; Lincoln, 1989). The 'seasonal' increase in FSH in the rams under investigation co-ordinated with the increases in testicular size and inhibin, which were positively correlated with each other. A sequential relationship was noted, with FSH increasing a few weeks before scrotal circumference and inhibin. For both groups, scrotal circumference and inhibin were more highly correlated with FSH a month earlier than with concurrent FSH. In individual rams, FSH peaks generally preceded those in scrotal circumference (94%) and inhibin (63%). Later seasonal peaks and decreases in inhibin relative to FSH appear to be characteristic of adult rams of most breeds of sheep (Lincoln *et al.*, 1990).

Although FSH participates in regulating developmental increases in inhibin secretion in rams, its exact role is uncertain. In an earlier study with adult DLS rams (Sanford *et al.*, 1993), the magnitude (amplitude and duration) of the 'seasonal' FSH increase appeared to determine the magnitude of the corresponding inhibin increase, yet in the present investigation (Expt 1), increases in inhibin of smaller

amplitude in group 2 than group 1 rams within each testicular cycle were not preceded by smaller increases in FSH. Nonetheless, the results of the present study indicate that fluctuations in FSH concentration *per se* may regulate inhibin secretion in specific stages of the 'seasonal' testicular cycle. For example, increases in inhibin in group 1 rams in redevelopment were accompanied by increases in FSH concentration, but not in the number of FSH receptors in the testis. Furthermore, when the testes were redeveloped, FSH and inhibin concentrations were both lower in group 2 than in group 1 rams, although the number of testicular FSH receptors was just as high in each group. Precisely defining the temporal, causal relationship between these two hormones will probably require measurement of bioactive FSH, as increases in inhibin in ram fetuses (Phillips *et al.*, 1992) and pubertal ewe lambs (Padmanabhan *et al.*, 1992) appear to be related more to bioactive than immunoactive FSH concentrations.

The ability of FSH to act on Sertoli cells in the testis of adult rats depends on the stage of the spermatogenic cycle within the seminiferous tubule. Differences in the extent of FSH stimulation between stages of the cycle are the result of differences in the number of FSH receptors present; expression of FSH receptors and concentrations of immunoactive and bioactive inhibin are all highest in stages XIII–III (Gnessi *et al.*, 1997; Simoni *et al.*, 1997). In the present study, the number of membrane-bound FSH receptors in the testes (number per Sertoli cell) of adult rams increased by an estimated 70% during recrudescence (Expt 1). Several experimental observations indicate that changes in FSH receptors *per se* during the 'seasonal' testicular cycle are central to FSH regulation of inhibin secretion. Firstly, inhibin concentration across the four stages of the 'seasonal' cycle was positively correlated with FSH receptor binding, but not with FSH concentration. Secondly, in group 1 rams, the increase in inhibin to peak concentrations as the testes completed redevelopment occurred as the number of FSH receptors continued to increase and FSH concentration began to decrease. Thirdly, although FSH concentrations in group 2 rams were lower than normal when the testes were fully redeveloped, the number of FSH receptors reached a maximum as usual and inhibin concentration remained relatively high. Although the evidence favours FSH and its cognate receptor system as a major modulator of inhibin secretion in rams, other controls may be just as important. In adult male mice made deficient in FSH receptors via targeted disruption of the FSH receptor gene, testicular content and blood concentration of inhibin are reduced by only 50% (Dierich *et al.*, 1998; Abel *et al.*, 2000).

In addition to seasonal increases in FSH concentration and number of FSH receptors, an increase in the abundance of the mRNA encoding the FSH receptor gene was observed (Expt 2). These data support the assumption that in males of photoperiodic species (for example, rams, Djungarian hamsters and quail), testicular recrudescence is sustained by increases in the number of FSH receptors induced by parallel increases in FSH secretion (Barenton and Pelletier,

1983; Simoni *et al.*, 1997). Although both the number of FSH receptors and abundance of FSH receptor mRNA increased during recrudescence, the values were not strongly correlated. This finding may be explained by the considerable turnover and recycling of membrane-bound receptors that occurs in Sertoli cells (Bardin *et al.*, 1994), such that estimates of FSH binding reflect only functional receptors at the cell surface, not total receptor protein and, thus, product of extant mRNA. It should also be recognized that FSH binding was expressed per testis and accurately reflects the number of membrane receptors present per Sertoli cell, whereas mRNA was corrected for number of all (somatic and germ) cells. As testes redevelop, the number of Sertoli cells remains constant, but the number of germ cells increases significantly. This 'dilution' of Sertoli cells implies that the measured increase in mRNA content during testicular redevelopment was underestimated. Although our correlative results indicate that FSH upregulates its own receptor in rams, other factors such as paracrine interaction between germ cells and Sertoli cells are probably involved (Simoni *et al.*, 1997), thereby influencing inhibin secretion and other FSH-dependent Sertoli cell functions. The most striking evidence for this in the present study is that the number of FSH receptors was normal in the redeveloped and regressing testes of group 2 rams (Expt 1), despite unusually low FSH secretion.

The possibility that testosterone is part of the paracrine system regulating inhibin secretion in rams was examined using passive immunization against oestradiol (Expt 2), enabling long-term, selective control of testosterone secretion under *in vivo* conditions (Sanford, 1987, 1989; Sanford *et al.*, 1991). Low-level immunization for 3 weeks produced moderate increases in basal testosterone concentrations in both the non-breeding (1.0 ng ml^{-1}) and breeding (4.5 ng ml^{-1}) seasons, and in mean testosterone concentration in the breeding season (8.5 ng ml^{-1}). The amplitude of episodic testosterone secretion in immunized rams was nearly double that in controls (Price *et al.*, 2000), probably contributing to the stronger testosterone signal, as Sertoli cells in ram testis appear to respond to individual androgen pulses (Monet-Kuntz and Terqui, 1985). Increases in testosterone concentration within the testes in the vicinity of androgen target cells would probably have been at least 30-fold higher (Voglmayr *et al.*, 1980; Tilbrook *et al.*, 1991). As described by Price *et al.* (2000), increases in testosterone secretion in immunized rams were due in part to increased expression of steroidogenic acute regulatory protein (StAR) and entry of cholesterol into the steroidogenic pathway. Importantly, increases in testosterone secretion occurred independently of changes in LH and FSH. Neither episodic LH release (Price *et al.*, 2000) nor LH receptor binding per testis (for example, breeding season: control rams, $119.9 \pm 18.4 \text{ pmol l}^{-1}$; immunized rams, $132.6 \pm 16.3 \text{ pmol l}^{-1}$; L. M. Sanford, D. G. Leggee and T. A. Yarney, unpublished) was affected by immunization. This finding should eliminate the possibility of any LH-induced paracrine effect of Leydig cells on inhibin secretion by

Sertoli cells not mediated by testosterone (Bardin *et al.*, 1989; Risbridger and de Kretser, 1989). Similarly, in keeping with our earlier studies, oestradiol immunization did not alter FSH secretion or the number of FSH receptors in the testis.

Circulatory concentrations of immunoactive inhibin in treated rams were not altered by the comparatively small increases in testosterone concentration when the testes were regressed (low FSH and inhibin) or by the more substantial increases in testosterone concentration when the testes were developed (high FSH and inhibin). These results are consistent with those of rat Sertoli cells in culture showing that testosterone does not influence basal or FSH-stimulated immunoactive inhibin secretion (Bicsak *et al.*, 1987; Toebosch *et al.*, 1989; Lampa *et al.*, 1999), although one study indicates that testosterone may suppress inhibin at low FSH concentrations (Morris *et al.*, 1988). The absence of an effect of testosterone on immunoactive inhibin in rams, both in the short-term (Tilbrook *et al.*, 1991) and long-term (present study), implies that the positive relationship between these two hormones during the seasonal testicular cycle is not causal. However, several investigators have suggested that testosterone may affect the secretion of bioactive inhibin (Steinberger, 1981; Verhoeven and Franchimont, 1983; Au *et al.*, 1986), possibly by regulating post-transcriptional events involving the processing, assembly and glycosylation of the inhibin subunits (Toebosch *et al.*, 1989). Whether the synthesis of inhibin A (or inhibin B) is modulated by testosterone in rams is not known.

In conclusion, this investigation provides new insights into the roles of FSH and testosterone in regulation of inhibin secretion during the seasonal testicular cycle of adult rams. Our experimental findings indicate that: (i) initial increases in immunoactive inhibin during testicular redevelopment are caused primarily by corresponding increases in FSH secretion, but that as development proceeds, FSH action is furthered by Sertoli cells acquiring additional FSH receptors; (ii) increases in functional, membrane-bound FSH receptors later in recrudescence are a result of FSH induction of gene expression for these receptors; and (iii) although FSH and its cognate receptor system play a central role, testosterone is at best a minor modulator of immunoactive inhibin secretion in rams, although the possibility of testosterone involvement in specific regulation of dimeric inhibin cannot be eliminated.

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