Production of inhibin A not B in rams: changes in plasma inhibin A during testis growth, and expression of inhibin/activin subunit mRNA and protein in adult testis

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Previous studies have shown that changes in the plasma concentrations of immunoreactive inhibin measured by radioimmunoassay occur in parallel with growth and regression of the testes during a reproductive cycle in adult Soay rams induced by exposure to an artificial lighting regimen of alternating 16 week periods of long days and short days. With the development of new two-site ELISAs for sheep inhibin A and inhibin B, we have re-examined the relationship between FSH and dimeric, biologically active inhibin in the reproductive cycle in adult Soay rams. No signal was generated by sheep testicular extract, ram or ewe plasma, or sheep ovarian follicular fluid in the inhibin B ELISA. In contrast, ram plasma contained significant activity in the inhibin A ELISA, which diluted in parallel to the inhibin A standard, and was abolished by preincubation of ram plasma with monoclonal antibodies specific for the βA, but not the βB, subunit. These results indicate that the ram is the first adult male mammalian species identified to date in which the testes produce and secrete dimeric inhibin A and not inhibin B. Northern blot analysis and immunocytochemistry confirmed the presence of α, βA and βB inhibin/activin subunit mRNA and protein in the testes of adult rams. Changes in plasma inhibin A concentrations occurred in parallel with the growth and regression of the testes during the long day:short day:long day lighting regimen in adult Soay rams, confirming our previous observations with immunoreactive inhibin. During the growth phase of the testes in the first 8 weeks of exposure to short days there was a positive correlation between plasma FSH and inhibin A concentrations, indicating that during this phase the secretion of inhibin A is stimulated by FSH and that inhibin A did not act as a negative feedback hormone on FSH secretion. From week 8.5 to week 16.0 of exposure to short days, there was a negative correlation between FSH and testosterone concentrations, but not inhibin, indicating that when inhibin concentrations are high, testosterone acts as the negative regulator of FSH secretion. Thus, in intact adult rams, when the testes are fully active it appears that inhibin A may sensitize the pituitary to the negative feedback effects of testosterone, at which time they act synergistically to maintain plasma concentrations of FSH.

Introduction

Inhibin is a dimeric glycoprotein, produced by the gonads, which suppresses FSH secretion by a direct action on the pituitary and is secreted as heterodimers of a common α subunit with either a βA subunit forming inhibin A, or a βB subunit forming inhibin B (Ying et al., 1988). The ovary produces both inhibin A and B, whereas the testes in all adult males studied to date (men: Illingworth et al., 1996; chimpanzees: Kondo et al., 2000; rhesus monkeys: Mann et al., 1997; Plant et al., 1997; rats: Woodruff et al., 1996; mice: Kananen et al., 1996; hamsters: Jin et al., 2001) secrete inhibin B and there is no evidence of the presence of inhibin A in plasma. However, during development of the testes and in neonatal life, the testes do express mRNA and protein for all three inhibin subunits, α, βA and βB (rats: Tena-Sempere et al., 1999; sheep: Jarred et al., 1999; men: Majdic et al., 1997; Anderson et al., 1998a,b). In rats, expression of the inhibin βA gene is lost before puberty (Tena-Sempere et al., 1999).

Initial studies using ovine, bovine or pig ovarian follicular fluid as a source of bioactive inhibin showed that inhibin suppressed FSH in adult males (Tilbrook and Clarke, 1995), although the effects of inhibin were reduced or absent in adult rats (Culler and Negro-Villar, 1988). More recently, studies using recombinant inhibin A, the only...
Inhibin available in sufficient quantities, confirmed the inhibin-induced suppression of FSH secretion in adult rhesus monkeys (Ramaswamy et al., 1998) and rams (Tilbrook et al., 1993a) through a direct action at the pituitary (Tilbrook et al., 1993b). Furthermore, studies in castrated rams have indicated that inhibin acts synergistically with testosterone to maintain the normal feedback regulation of FSH secretion (Tilbrook and Clarke, 2001).

In previous studies it was shown that the seasonal changes in testis growth and regression in rams of various breeds were associated with parallel changes in the plasma concentrations of immunoreactive inhibin (Lincoln and McNeilly, 1989; Lincoln et al., 1990). The radioimmunoassay used in these studies was directed against the N-terminus of the αC subunit of inhibin (McNeilly et al., 1989) and, as such, detected inhibin A, B and free α subunit (McNeilly et al., 1994), and would not necessarily reflect the changes in the plasma concentrations of the bioactive dimeric inhibin forms. More recently, O’Brien et al. (1996) and Knight et al. (1998) have developed an ELISA that specifically measures inhibin A in sheep. During the development of the ELISA, standards were made up in ram plasma as a control in the expectation that ram plasma would provide a matrix devoid of inhibin A. Unexpectedly, ram plasma was found to contain substantial amounts of material that crossreacted, which, because of the intrinsic specificity of the ELISA, should only be inhibin A. This unexpected finding prompted us to investigate the validity of this observation and to develop a sheep inhibin B ELISA. After confirmation that the inhibin A ELISA was detecting activity in ram plasma, changes in plasma concentrations of inhibin A during a light-induced testis growth and regression cycle in Soay rams were determined, and mRNA expression, and the presence and localization of the inhibin α, βA and βB subunits, were determined in the testes of sexually active adult rams. This work has been reported in brief by McNeilly et al. (2000).

Materials and Methods

Inhibin ELISAs and FSH and testosterone radioimmunoassays

The ELISA for inhibin A designed specifically for sheep was first described by O’Brien et al. (1996) and the method and specificity of the ELISA for inhibin A is described in more detail by Knight et al. (1998). In brief, the ELISA is based on the use of an immobilized monoclonal antibody (E4) raised against synthetic peptides corresponding to the amino acid sequence 82–114 of the human and ovine βA subunit, and an αC-specific biotinylated monoclonal antibody (17329/H2) raised against synthetic peptides corresponding to the amino acid sequence 1–32 of the human αC subunit as detection antibody (Knight et al., 1998). Purified 32 kDa bovine inhibin A (Knight and Muttukrishna, 1994) diluted in ovariectomized ewe plasma was used as standard. Both standards and plasma samples were denatured by boiling in 6% (w/v) SDS before oxidation (Souza et al., 1997), as in the human inhibin A ELISA (Groome et al., 1994). After incubation of the assay in a 96-well plate, the plates were read using a Labsystems Multiskan MCC/340 plate reader (Life Science International, Basingstoke) at a wavelength of 492 nm. The sensitivity of the inhibin A ELISA was 30 pg ml–1 and the intra- and interassay coefficients of variation were both < 12%.

After denaturation and oxidation, samples were preincubated with 2 μg ml–1 of either βA (E4) or βB (12/13) monoclonal antibodies for 6 h at room temperature before addition to the inhibin A ELISA for determination of the specificity of the signal in ewe and ram plasma.

The inhibin B ELISA was identical to the inhibin A ELISA in format except that two monoclonal antibodies (12/13 or C5; Groome et al., 1997) which detected βB subunit within adult ram testes (see below) were used as capture antibodies in separate ELISAs with the biotinylated αC monoclonal antibody as detection antibody. Human inhibin B was used as standard and was detected using both formulations with a sensitivity of 30 pg ml–1.

Plasma concentrations of FSH (McNeilly et al., 1976) and testosterone (Lincoln and McNeilly, 1989) were measured by radioimmunoassay as described previously. Reagents used in the FSH radioimmunoassay were supplied by A. F. Parlow (NHPP, Harbor-UCLA, CA). Intra- and interassay coefficients of variation were < 10% for each radioimmunoassay, with a sensitivities of 0.1 ng USDA-oFSH-SIAFP-RP2 (AFP 4117A) ml–1 and 0.3 nmol testosterone l–1.

Animals, blood sampling and collection of tissues

These studies were conducted at the Marshall Building, Roslin, Edinburgh, UK and were approved and conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1996 of the United Kingdom.

Soay rams. A group of Soay rams (n = 6; aged 4–5 years) housed in light-controlled rooms were exposed to alternating 16 week periods of long (16 h light:8 h dark) and short (8 h light:16 h dark) days (Lincoln and Short, 1980). The animals were housed in individual pens and maintained on a diet of grass pellets, hay and water, which were available ad libitum. Blood samples (3 ml) were collected by jugular venepuncture from each animal twice a week and the diameter of the testes was recorded every 2 weeks over a 32 week period spanning a complete cycle of short and long days. Plasma was separated and stored at –20°C until assayed.

Cross-bred rams. Testes were obtained from sexually active and successfully breeding Merino × Scottish Blackface rams (n = 6; aged 4–5 years) kept outdoors on grass. The rams were killed by an overdose of sodium pentobarbitone anaesthetic (25 ml Euthatal; Rhone Merieux Ltd, Harlow). Two blood samples (5 ml) were collected by jugular venepuncture at day 2 before and immediately before death, and plasma was separated and stored at –20°C until assayed. A sample of each testis was collected and fixed in Bouin's
solution for 6 h at room temperature before processing into paraffin wax (Millar et al., 1993). A further sample of each testis was snap frozen and stored in liquid nitrogen for molecular analysis. The presence of spermatozoa was confirmed in each animal when the testes were dissected.

**Immunocytochemistry**

The same monoclonal antibodies specific for βA (E4: 0.1 μg ml⁻¹) and βB (12/13: 0.2 μg ml⁻¹; C5: 0.4 μg ml⁻¹) inhibin/activin used in the sheep inhibin A and B ELISAs, and a rabbit polyclonal antibody (ASM R146: 1:1000) raised against the N-terminal 1–32 amino acids of porcine αC inhibin previously characterized and used in a radioimmunooassay for sheep immunoreactive inhibin (McNeilly et al., 1989), were used for immunocytochemistry. Sections (5 μm in thickness) were stained as described previously after antigen retrieval by microwaving the sections (Thomas et al., 1995; Majdic et al., 1997). The specificity of these antibodies was controlled by using normal mouse or rabbit serum instead of primary antibodies and by pre-adsorption of the antibodies with the corresponding peptide. The monoclonal antibodies have been used for immunocytochemistry in rat, human (Majdic et al., 1997) and sheep testes (Thomas et al., 1995; Jarred et al., 1999). Images were captured into a Macintosh G4 computer using an Olympus Provis Image analysis system (Olympus Optical Co., London) equipped with a Kodak DCS420 camera (Eastman Kodak, Rochester, NY).

**Northern blot analysis**

Total RNA was extracted from all testes collected (n = 8) and 20 μg samples were loaded for northern blot analysis using cDNA probes labelled with [³²P]dCTP using a random-primed labelling kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfont). The probes were as follows: sheep inhibin α cDNA; gift from C. McDougal, Department of Obstetrics and Gynaecology, University of Edinburgh; sheep βA cDNA: full length clone KZ72, gift from S. Galloway, AgResearch Molecular Biology Unit, University of Otago, New Zealand (Fleming et al., 1992; 1995); sheep βB cDNA: full length clone, gift from R. Rodgers, University of Adelaide (Rodgers, 1991).

The efficiency of loading was measured by re-probing the membranes with a rat 18S ribosomal RNA oligonucleotide probe. The intensity of bands was quantified using a phosphorimaging system (425; Molecular Dynamics, Chesham) and mRNA values are expressed as corrected values relative to 18S rRNA.

**Analysis of results**

Changes in plasma concentrations of hormones were analysed by ANOVA and, when significant changes were confirmed, further analysis was undertaken using Fisher’s protected least significance difference test, using Statview statistical package (Statview 4.02; SAS Institute Inc., San Francisco, CA) and by paired t tests using SPSS for Macintosh (SPSS 10.07a; The Software MacKiev Company, Cupertino, CA). Linear correlations between group mean plasma concentrations of FSH, inhibin A and testosterone for weeks 0–8 and 8.5–16.0 under short days were calculated using Cricket Graph software (Cricket Graph Inc., Philadelphia, PA). These two periods were preselected to represent the growth and active phases of the photoperiod-induced reproductive cycle (Lincoln and Short, 1980).

**Results**

**Inhibin ELISA**

Dilutions of ovine follicular fluid and plasma were parallel to the bovine standard preparation (data not shown). Serial dilutions of ram plasma were also parallel to the inhibin A standard (Fig. 1). Recovery of standard added to ram plasma was 101 ± 2% (mean ± SEM, n = 10). Preincubation of ram plasma with the monoclonal antibody (12/13) to βB subunit did not affect the signal in ram plasma. In contrast, preincubation of ram plasma with the specific monoclonal antibody (E4) to the βA subunit abolished the signal in ram plasma completely (Fig. 1).

Although the inhibin B ELISA detected human inhibin B, there was no crossreaction with either ewe or ram plasma, and the small signal detected with dilutions of ovine and bovine follicular fluid or ram testicular extracts was calculated to be equivalent to the crossreaction of inhibin A with the monoclonal inhibin BB antibody (data not shown). No further studies were carried out with this ELISA.
Changes in plasma inhibin A, FSH and testosterone concentrations, and testis size

The changes in plasma concentrations of FSH, testosterone and inhibin A concentrations, and in testis size over the period from long days to short days and back to long days are shown (Fig. 2). The changes in plasma concentrations of inhibin A mirrored the changes in testis size (Fig. 2). The first significant (P < 0.05) increase in FSH concentrations at week 2 of short days preceded the first significant (P < 0.05) increase in testis size at week 4 of short days and in inhibin A concentrations at week 4.5 of short days, whereas testosterone concentrations only increased significantly (P < 0.05) above basal values at week 8 of short days. During the first 8 weeks of short days there was a highly significant (P < 0.001) positive correlation between FSH and inhibin A concentrations (r² = 0.938; Fig. 3a), but there was no correlation with testosterone concentrations (r² < 0.2; data not shown). Between week 8.5 and week 16.0 of short days there was no significant correlation between FSH and inhibin concentrations (r² < 0.1), but there was a highly significant (P < 0.001) negative correlation between FSH and testosterone concentrations (r² = 0.780; Fig. 3b). Maximum plasma concentrations of FSH occurred at about week 7 of short days, about 5 weeks before maximum concentrations of inhibin A and maximum testicular size were achieved (Fig. 2). Plasma FSH concentrations started to decrease from week 8 of short days onwards, coinciding with the first significant and sustained increase in plasma testosterone concentrations (Fig. 2).

Fig. 2. Changes in (a) testis diameter (○) and plasma concentrations of FSH (●) and (b) plasma concentrations of inhibin A (●) and testosterone (○) in adult Soay rams exposed to an alternating 16 week periods of long (16 h light:8 h dark; LD) or short (8 h light:16 h dark; SD) days. The growth phase of the testes continues from week 0 to week 10, and the testes become fully active from approximately week 12 to the onset of long days at week 16, which induces testis regression.

Fig. 3. Correlations between plasma concentrations of (a) FSH and inhibin A from week 0 to week 8 of exposure to short days (8 h light:16 h dark), and (b) FSH and testosterone from week 8.5 to week 16.0 of exposure to short days, in rams.
Inhibin/activin subunit mRNA

Northern blot analysis indicated the presence of single mRNA transcripts for the inhibin/activin α (1.3–1.6 kb) and βA (7–8 kb) subunits in all six adult ram testes examined (Fig. 4a). Two transcripts were present for the βB subunit mRNA (approximately 3 and 4 kb) and quantitative analysis indicated that they were of equivalent magnitude in each sample (Fig. 4a).

Immunocytochemistry

All inhibin subunit proteins were present in the seminiferous tubules in all six adult testes examined (Fig. 4b). Immunostaining for α and βA subunits was intense in Sertoli cells, whereas βB subunit was present but staining was less intense. All three subunits were associated with germ cells, and the distribution for α and βA subunits was directly comparable. Inhibin α subunit staining was also present in residual bodies. βB subunit protein was also associated with germ cells and, in many cases, intense staining surrounded germ cells at all stages. All subunit proteins showed weak staining in Leydig cells. There was no staining after pre-adsorption of each antibody with the respective peptides used as immunogens (Fig. 4b).
Discussion

The present study has identified the first mammal to date in which the adult testis secretes inhibin A and not inhibin B into the circulation. Until this observation it had been shown that, in adult male mice, rats, hamsters, rhesus monkeys, chimpanzees and men, the only form of inhibin present in plasma was inhibin B. Previously it had been shown that the sheep inhibin A ELISA did not crossreact with activin A, activin B, inhibin B, inhibin α subunit, follistatin-288 and alpha2-macroglobulin (Knight et al., 1998). In the present study, pre-adsorption of ram plasma with the monoclonal antibody specific for the inhibin βA, but not the βB subunit, completely abolished the signal in the ELISA. Furthermore, although an apparently viable ELISA for inhibin B was established using monoclonal antibodies that had been used in other ELISAs for inhibin B and for immunocytochemistry in sheep testis (Jarred et al., 1999; present study), no signal was generated with either testis extract or ram plasma, or ovine ovarian follicular fluid, a rich source of inhibin B in other species (Groome et al., 1996). Indeed, Knight et al. (1998a) concluded that there was little, if any, inhibin B in sheep ovarian follicular fluid. Thus, it appears that the inhibin A ELISA is detecting an inhibin A signal in ram plasma, which dilutes parallel to the inhibin A standard.

Both the α and βA subunits must be expressed in adult ram testis to generate inhibin A. In the testis of sheep fetuses, mRNA and proteins for α, βA and βB subunits are all expressed (Jarred et al., 1999). In the present study, the presence of all three inhibin/activin subunits in adult ram testis was confirmed for mRNA by northern blot analysis and for protein by immunocytochemistry. The mRNAs were of the expected sizes (Rodgers et al., 1989; Tena-Sempere et al., 1999). All three inhibin/activin subunits were present mainly in Sertoli cells, as in the testis of sheep fetuses (Thomas et al., 1995; Jarred et al., 1999). There was only weak staining of interstitial cells for all subunits, indicating a limited activity of Leydig cells in generating inhibin A. Maximum staining for βB subunit occurs in the Leydig cells in adult golden hamsters (Jin et al., 2001) and stallions (Nagata et al., 1998), and, in these species, the inhibin B in plasma may originate from Leydig cells. In other species for which data are available, inhibin B appears to originate from the Sertoli cells, which have maximum α and βB subunit mRNA and protein contents (rat: Klaij et al., 1994; Tena-Sempere et al., 1999; human: Anderson et al., 1998a). The testes in adult rats (Meunier et al., 1988; Tena-Sempere et al., 1999) and hamsters (Jin et al., 2001) do not express βA subunit. The situation in men is not clear; although no inhibin A appears to be produced and there is no inhibin A in plasma or seminal plasma, the βA subunit protein has been detected, mainly in Leydig cells (Anderson et al., 1998b), and the testes produce activin A, the βA–βA dimer (Anderson et al., 1998b; Muttukrishna et al., 2001). Thus, the expression of α and βB subunits is compatible with the secretion and presence in plasma of inhibin B in most adult males, whereas the presence of the βA subunit in the testes of men results in the production of only activin A, and no inhibin A.

It is unclear how the ram testis, while expressing the α, βA and βB subunits, only appears to produce inhibin A and not inhibin B. As all the subunits appear in the same types of cell, this finding indicates that there are specific intracellular pathways that determine that α combines only with the βA subunit to produce inhibin A for export into the circulation. It remains possible that small amounts of inhibin B are produced that act within the testes in a paracrine manner and are not released into the peripheral circulation. The sheep pituitary is much less sensitive to the effects of inhibin B (5%) than inhibin A (100%) in terms of suppression of FSH secretion (Robertson et al., 1997) and inhibin A is fully active in adult rams and will suppress FSH secretion (Tilbrook et al., 1993a). These observations, together with the finding that rams produce inhibin A rather than inhibin B, are consistent with the concept that inhibin A is the critical feedback inhibin in rams, a situation paralleled in ewes, in which the ovaries also appear to produce only inhibin A and not inhibin B, as no inhibin B could be detected in sheep ovarian follicular fluid in the present study.

The change in the pattern of secretion of inhibin A during the light-induced growth and regression cycle of the testes is very similar to our previous reports on changes in immunoreactive inhibin in a range of breeds of ram (Lincoln and McNeilly, 1989; Lincoln et al., 1990). The positive correlation between FSH and inhibin A concentrations in plasma during the growth phase of the testes during exposure to short days is similar to that observed during early puberty in boys (Crofton et al., 1997). During this time, there is no relationship between the changes in testosterone, and either inhibin or FSH. In contrast, the decrease in plasma FSH concentrations from week 8.5 of short days onwards was negatively correlated with testosterone concentrations. Indeed, FSH concentrations peak and only begin to decrease when there is a significant and sustained increase in testosterone secretion. This coincides with a period of high frequency low amplitude pulses of GnRH (Lincoln, 1978a), which may represent a reduced positive drive to FSH synthesis and secretion (Padmanabhan and McNeilly, 2001).

The results of the present study indicate that inhibin A has little negative feedback effect on FSH secretion during the transition from inactive (non-breeding season; long days) to active (breeding season; short days) testis function, and that the major regulator of FSH secretion in rams when the testes are fully active and secreting inhibin A is testosterone. Previous studies in castrated rams have shown that both inhibin and testosterone act directly at the pituitary to suppress FSH secretion (Tilbrook et al., 1993b), effects which occurred independently of hypothalamic input and GnRH pulse frequency (Tilbrook et al., 2001). Inhibin A alone suppressed FSH secretion during both the non-breeding and breeding season, whereas testosterone

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alone had a limited effect during the breeding season, but was without effect in the non-breeding season (Tilbrook et al., 1993a, 1999). However, when testosterone was given with inhibin there was a substantial synergistic effect in suppressing FSH secretion in castrated rams (Tilbrook et al., 1999).

These effects of inhibin and testosterone treatment discussed above were studied in castrated rams during mid-breeding or mid-non-breeding seasons. The implication that inhibin is a more potent negative feedback regulator of FSH than testosterone in rams may not be incompatible with the observation that, in intact Soay rams during the transition from the non-breeding to the breeding season, testosterone is more important than inhibin. In the normal cycle of growth and regression of the testes in rams, the initial drive during short days is from GnRH in the form of slow frequency, high amplitude pulses, which increases FSH and stimulates testis growth (Lincoln, 1978b), with a resultant increase in activation of Sertoli cells and production of inhibin A. In the presence of only low concentrations of testosterone, inhibin A has a limited effect as a negative feedback regulator of FSH secretion, when the positive drive from GnRH appears to be optimal for FSH production (Lincoln, 1978a; Padmanabhan and McNeilly, 2001). From about week 8 of short days when the testes have grown substantially and are becoming active, the pulse frequency of GnRH increases (Lincoln, 1978b). The resultant increase in LH pulse frequency stimulates a continued increase in testosterone secretion, which synergizes with inhibin to become an effective negative feedback signal to control FSH secretion, as reported in castrated rams treated with testosterone or inhibin or both (Tillbrook et al., 1999).

Changes in the expression of the inhibin receptor may occur throughout the growth and regression cycle of the testes, but this cannot be investigated at present because the structure of the sheep inhibin receptor is not known. A specific inhibin binding site has been reported in sheep pituitaries (Hertan et al., 1999) that has a much higher affinity for inhibin A than inhibin B (Robertson et al., 1997), but which is not one of the putative inhibin receptors, betaglycan (Lewis et al., 2000) or P120 (Chong et al., 2000), reported to date. Further studies on the changes in inhibin, androgen and possibly oestrogen receptors within the pituitary gonadotropes, and the activation of the pathways suppressing FSH synthesis and secretion, will be required to elucidate the mechanisms underlying the regulation of FSH secretion by inhibin and testosterone during the growth and regression phases of the testicular cycle in rams.

In conclusion, the present study has described, for the first time, a male mammal in which the testes produce inhibin A and not inhibin B. The pattern of secretion of inhibin A in rams parallels the growth of the testes and during this phase there is a positive relationship between plasma concentrations of FSH and inhibin A. At this stage, in the presence of low plasma concentrations of testosterone and maximum positive drive from GnRH, inhibin has a limited action as a negative regulator of FSH secretion. Once the testes achieve maximum size and testosterone concentrations increase, plasma concentrations of FSH begin to decrease rapidly in inverse relationship to testosterone. Inhibin A concentrations then decrease more slowly. Thus, in intact adult rams, when the testes are fully active it appears that inhibin A may sensitize the pituitary to the negative feedback effects of testosterone, at which time they act synergistically to maintain plasma concentrations of FSH.

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