Studies on the testicular source of inhibin and its route of secretion in rams: failure of the Leydig cell to secrete inhibin in response to a human chorionic gonadotrophin/LH stimulus

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ABSTRACT

To determine whether Leydig cells produce inhibin in the ram, Leydig cells were stimulated by administering human chorionic gonadotrophin (hCG) or raising the levels of endogenous LH by an injection of gonadotrophin releasing hormone (GnRH). Plasma concentrations of testosterone increased in the 72 h after either a single injection (P<0.05) or two injections (P<0.01) of hCG. Plasma concentrations of inhibin were not significantly influenced by either one or two injections of hCG. Administration of GnRH (1 μg) caused an 11-fold increase in plasma concentrations of LH but did not influence concentrations of inhibin in either the jugular or testicular veins (pampiniform plexus). In contrast, concentrations of testosterone were increased by about fourthfold in both jugular (P<0.01) and testicular (P<0.05) veins. The concentrations of inhibin in the testicular vein were 1.3-fold higher than in the peripheral plasma (P<0.05) both before and following treatment with GnRH whereas the concentrations of testosterone were 18- to 21-fold greater than in peripheral concentrations.

In view of the difference in concentrations of inhibin between testicular and jugular veins, in a further experiment a sample was taken from the jugular vein, a vein located in the tunica vasculosa of the testis (testicular vein) and from a vein (spermatic vein) and lymph vessels located in the spermatic cord. The mean (± S.E.M.) concentrations of inhibin were highest in the testicular lymph (45.9±4.21 μg/l; P<0.001) compared with the peripheral (4.1±0.31 μg/l), spermatic (8.0±1.17 μg/l) or testicular (6.4±0.82 μg/l) plasma. Plasma concentrations of inhibin were significantly higher in the spermatic vein than in the testicular vein (P<0.05) and jugular vein (P<0.01), and concentrations of inhibin in the testicular vein were significantly (P<0.05) higher than in the jugular vein. There were no significant differences in the concentrations of testosterone in the spermatic vein, testicular vein or testicular lymph but the concentrations of testosterone in the peripheral plasma were significantly (P<0.05) less than in the testicular plasma or lymph.

These results suggest that, in the ram, the Leydig cell does not respond to hCG or endogenous LH by secreting inhibin or by influencing other cells within the testis to secrete inhibin within the time-frame of these experiments. The low testicular to jugular differences in the concentration of inhibin and the high concentrations of inhibin in the testicular lymph suggest that the lymph may be an important route of secretion of inhibin from the testis in the ram.


INTRODUCTION

Inhibin is a glycoprotein hormone consisting of two dissimilar subunits, termed α and β, joined by disulphide bonds, which inhibits the production and/or secretion of the gonadotrophins from the pituitary, preferentially follicle-stimulating hormone (FSH) (Burger & Igarashi, 1988). The β subunit of inhibin exists in two forms, βA and βB, and combines with the α subunit to produce inhibin A and inhibin B (Burger & Igarashi,
In the male it has been generally accepted that the Sertoli cells are the major testicular source of inhibin. This is due largely to studies with cultures of Sertoli cells (Steinberger & Steinberger, 1976; Le Gac & de Kretser, 1982; Verhoeven & Franchimont, 1983; Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1986; Bicsak, Vale, Vaughan et al. 1987; Ultee-van Gessel & de Jong, 1987; Morris, Vale, Cappel & Bardin 1988; Roberts, Meunier, Sawchenko & Vale, 1989; Toebosch, Robertson, Klaïj et al. 1989) and seminiferous tubules (Eddie, Baker, Dulmanis et al. 1978; Gonzales, Risbridger & de Kretser, 1988; Bhasin, Krummen, Swerdloff et al. 1989; Gonzales, Risbridger, Hodgson et al. 1989). Nevertheless, recent studies in men and rats have indicated that the Leydig cells may also produce inhibin in the testis. For instance, McLachlan, Matsumoto, Burger et al. (1988a) suggested that normal inhibin secretion in men requires the combined action of both FSH and luteinizing hormone (LH) because selective suppression of FSH to undetectable levels by administration of human chorionic gonadotrophin (hCG) resulted in a 30% fall in inhibin whereas a 60% fall occurred when both FSH and LH were suppressed with exogenous administration of testosterone. Stimulation of the function of Leydig cells by administration of hCG was found to increase the levels of serum inhibin in men (McLachlan, Matsumoto, Burger et al. 1988b; Fingscheidt & Nieschlag, 1989; Burger, Tiu, Bangah & de Kretser, 1990; McLachlan, Finkel, Brenner & Snyder, 1990) and the levels of inhibin in testicular interstitial fluid (Sharpe, Kerr & Maddocks, 1988) and serum (Drummond, Risbridger & de Kretser, 1989) of adult male rats. This effect in rats was abolished when the Leydig cells were destroyed by treatment with the Leydig cell cytotoxic ethane dimethane sulphonate (EDS) suggesting that the Leydig cells were the source of the inhibin stimulated by hCG (Drummond et al. 1989). Each of these effects may be explained by the recent demonstration that Leydig cells from adult male rats express mRNA for the α subunit of inhibin and produce inhibin which is biologically and immunologically active (Risbridger, Clements, Robertson et al. 1989). In addition, Roberts et al. (1989) provided evidence that the Leydig cells in the immature and adult rat contained inhibin and mRNA for its subunits but could not stimulate the production of inhibin from Leydig cells by administration of LH-like hormones in the adult rat. A recent report has also documented the presence of activin, which is a dimer of the β subunits of inhibin, in a tumour-derived line of Leydig cells from male mice (Lee, Mason, Schwall et al. 1988) and these results were supported by an immunocytochemical study of the rat testis (Shaha, Morris, Chen et al. 1989).

In an immunohistochemical study of the ram testis intense staining of inhibin was obtained in the seminiferous epithelium while the Leydig cells were also occasionally stained but it was not clear whether this staining was a result of production of inhibin by the Leydig cells or of a consequence of binding or uptake of inhibin produced by Sertoli cells (Veeramachaneni, Schanbacher & Amann, 1989). This paper reports the results of studies designed to determine whether the Leydig cells of the ram secrete inhibin and to investigate the major route of secretion of inhibin from the testis in this species.

**MATERIALS AND METHODS**

**Animals**

Adult Romney Marsh rams were used in each of these experiments which were conducted during the non-breeding season for this breed of sheep. Between experiments the rams were grazed on natural pastures at the Victorian Institute of Animal Science, Werribee, Victoria, Australia. For the duration of experiment 1 the rams were penned individually in an animal house and offered a ration consisting of 93-2% dry matter containing 11-2% crude protein with an estimated metabolizable energy content of 6·8 MJ/kg dry matter. Water was available **ad libitum**. Experiments 2 and 3 were conducted under sterile surgical conditions with the rams under halothane anaesthesia (May and Baker, West Footscray, Victoria, Australia) after induction with pentobarbitone sodium (Parke-Davis, Sydney, NSW, Australia). Prior to surgery, and for 24 h afterwards, the rams were kept in pens in the surgical complex at the Victorian Institute of Animal Science, Werribee and returned to grazing once they were deemed to have fully recovered from the surgical procedures.

The care and experimental use of the animals in these experiments conformed with the requirements of the **Australian Prevention of Cruelty to Animals Act** (1986) and the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and with the Guidelines on the Use of Living Animals in Scientific Investigations, edition 2, published by the Biological Council, London.

**Experimental procedure**

**Experiment 1**

Eighteen rams were allocated to three groups of six and were given either an s.c. injection of sterile saline (group 1), an s.c. injection of 1000 IU hCG (Pregnyl; Organon, Lane Cove, NSW, Australia; group 2) or two s.c. injections of hCG separated by 11 h (group 3). Blood samples were withdrawn via indwelling catheters (Dwellcath; Tuta Laboratories, Lane Cove,
NSW, Australia), from a jugular vein of each ram every 30 min for 1 h before treatment and for 3 h following the commencement of treatment, then at 6, 9 and 11 h after commencement of treatment. The rams in group 3 were then injected s.c. with a further 1000 IU hCG and sampling was continued in all rams at 30-min intervals for 2 h. Blood samples (5 ml) were also taken from each ram 24, 27, 30, 48, 51, 54 and 72 h after the commencement of treatment. The blood was collected into heparinized tubes and the plasma harvested and stored at −15 °C for subsequent determinations of concentrations of inhibin and testosterone.

Experiment 2
In four rams, the spermatoc cord and pampiniform plexus were exposed by means of a midline scrotal incision. A vein from the pampiniform plexus in the spermatic cord and a jugular vein were cannulated (Dwellcath), and matched samples were collected from each vein every 5 min over 90 min. Each ram was injected i.v. with 1 μg gonadotrophin-releasing hormone (GnRH; Auspep, South Melbourne, Victoria, Australia) after 30 min of sampling. Blood from the pampiniform plexus vein drained from the catheter by natural flow into a heparinized tube. The plasma was separated and stored at −15 °C for determination of concentrations of inhibin, testosterone and LH.

Experiment 3
The testis and spermatic cord were exposed in six rams by a scrotal incision. A sample of blood was taken from the jugular vein, a vein located on the surface of the testis in the tunica vasculosa, hereafter called a testicular vein, and from a spermatic vein close to the inguinal ring. Testicular lymph was also collected by puncture of the lymph vessels in the spermatic cord. All samples were collected into heparinized tubes, centrifuged and the plasma was stored at −15 °C for determination of the concentrations of inhibin and testosterone.

Radioimmunoassays
Inhibin
Immuoactive inhibin was measured using a double-antibody radioimmunoassay based on an antiserum (1989) raised against bovine 31 kDa inhibin and using iodinated 31 kDa bovine inhibin as tracer (Robertson, Hayward, Irby et al. 1988). This assay has been validated for measurement of ovine inhibin (Findlay, Clarke & Robertson, 1990) and cross-reacts 288% with pro-α, a product of the α subunit of inhibin (Robertson, Giacometti, Fouls et al. 1989). No significant cross-reactivity occurs to activin A, transforming growth factor β (TGFβ) and Mullerian-inhibiting substance. The sensitivity of this assay was 0.2 μg/l and the intra- and interassay coefficients of variation were 6.6 and 4.0% respectively.

Testosterone
Plasma concentrations of testosterone were measured, after ether extraction, as described by Risbridger, Kerr, Peake & de Kretser (1981) using [3H]testosterone (Dupont Australia Ltd, North Sydney, NSW, Australia) as ligand and an antiserum kindly provided by Dr R. Cox (Commonwealth Scientific and Industrial Research Organisation, Blacktown, NSW, Australia). Eight assays for testosterone were conducted for these experiments. The intra-assay coefficients of variation varied from 3.0 to 6.8% and the interassay coefficient of variation was 5.6%. The sensitivity of the assays for testosterone ranged from 0.17 to 0.24 nmol/l.

LH
Plasma concentrations of LH were measured by radioimmunoassay as described by Lee, Cumming, de Kretser et al. (1976). The standard used was NIH LH S18 (NIDDK, Baltimore, MD, U.S.A.) and the sample dose was 300 μl. All samples were measured in a single assay with a sensitivity of 0.26 μg/l. The maximum point of precision was 6.5% at 7.7 μg/l and the intra-assay coefficient of variation was 7.1%.

Statistical analysis
In experiment 1, analysis of variance was used to evaluate the effects of the injections of saline or hCG on concentrations of plasma inhibin and testosterone over time. Paired comparisons were made using least-significant differences. For both testosterone and inhibin, the mean concentrations for the period of sampling prior to treatment and 12, 24, 48 and 72 h after treatment had commenced were compared. In experiment 2, for both the jugular and pampiniform plexus veins, the mean concentrations of plasma inhibin, testosterone and LH were compared before and following the injection of GnRH using analysis of variance. Comparisons were also made between the mean concentrations of each of these hormones in the jugular and pampiniform plexus veins. In experiment 3, analysis of variance was used to compare the mean concentrations of inhibin and testosterone in plasma from the jugular vein, spermatic vein, testicular vein and from the testicular lymph vessels.

RESULTS
Experiment 1
Before the commencement of treatment there were no significant differences in the concentrations of plasma testosterone in all groups. Injection of saline (group 1) had no significant effect on the concentrations of
plasma testosterone (Fig. 1). Treatment with hCG caused a significant increase in the concentrations of plasma testosterone both in the rams given a single injection ($P<0.05$) and those given two injections ($P<0.01$) of 1000 IU hCG (Fig. 1). In the rams given a single injection of hCG (group 2) plasma concentrations of testosterone were significantly ($P<0.05$) increased within 12 h and continued to increase until 48 h after the injection when they were sixfold higher than before injection (Fig. 1). The concentrations of plasma testosterone were similar at 48 and 72 h after injection of hCG. Similarly, in the rams given two injections of hCG (group 3) the plasma concentrations of testosterone increased significantly ($P<0.01$) within 12 h of the first injection and continued to increase over 48 h, remaining elevated at 72 h and representing a 14- to 17-fold increase (Fig. 1). Throughout the experiment there was no difference in the plasma concentrations of testosterone of rams receiving one (group 2) or two (group 3) injections of hCG and these concentrations were always significantly ($P<0.05$) higher than in the saline-treated rams (group 1) following treatment.

Treatment with saline had no significant effect on concentrations of plasma inhibin, and there were no differences in the plasma concentrations of inhibin between the groups of rams. In contrast to the effects on testosterone, neither one nor two injections of hCG influenced plasma concentrations of inhibin (Fig. 1).

**Experiment 2**

The plasma concentrations of inhibin, testosterone and LH are shown in Table 1 and a representative example from one ram is shown in Fig. 2. Treatment with GnRH significantly increased plasma LH (Table 1 and Fig. 2) by 10- to 11-fold in the jugular vein ($P<0.05$) and 15-fold in the pampiniform plexus vein ($P<0.05$). However, GnRH did not significantly influence concentrations of inhibin in either the peripheral or testicular plasma (Table 1 and Fig. 2). In contrast, concentrations of plasma testosterone (Table 1 and Fig. 2) were increased by almost fourfold ($P<0.01$) in the jugular vein and by a similar magnitude in the pampiniform plexus vein ($P<0.05$) following treatment with GnRH.

The concentrations of inhibin in the plasma from the pampiniform plexus were 1-3-fold higher than in the peripheral plasma ($P<0.05$) both before and after treatment with GnRH. Concentrations of testosterone in the plasma from the pampiniform plexus vein were about 18-fold higher than those in peripheral plasma but this difference was not significant ($P=0.07$). Following treatment with GnRH, concentrations of testosterone in the testicular plasma were significantly higher than in the peripheral plasma (20-78-fold difference; $P<0.05$). Plasma concentrations of LH in the jugular and pampiniform plexus veins did not differ significantly before the injection of GnRH but, following injection, the concentrations in the pampiniform plexus vein were significantly ($P<0.05$) lower than those in the jugular vein.

**Experiment 3**

There were significant differences ($P<0.01$) in the concentrations of inhibin between each of the veins sampled (Table 2). The highest levels were in the testicular lymph ($P<0.001$) compared with the testicular or peripheral plasma (Table 2). Concentrations of inhibin were significantly higher in the spermatic vein than in the testicular vein ($P<0.05$) and jugular vein.
TABLE 1. Plasma concentrations of inhibin and testosterone in the jugular vein (JV) and pampiniform plexus vein (PPV) of rams before and after the i.v. injection of 1 µg gonadotrophin-releasing hormone (GnRH). Values are means ± S.E.M. of four rams.

<table>
<thead>
<tr>
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<th>Inhibin (µg/l)</th>
<th>Testosterone (nmol/l)</th>
<th>LH (µg/l)</th>
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<tr>
<td></td>
<td>JV</td>
<td>PPV</td>
<td>JV</td>
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<tr>
<td>Before GnRH</td>
<td>5·65 ± 0·17a</td>
<td>7·07 ± 0·45a</td>
<td>4·61 ± 1·94*</td>
</tr>
<tr>
<td>After GnRH</td>
<td>5·18 ± 0·23a</td>
<td>7·36 ± 0·65a</td>
<td>16·6 ± 4·13a</td>
</tr>
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For each of inhibin, testosterone and LH, significant differences between means are illustrated by different superscripts: a,b,c,dP < 0·05, *a,b,c,dP < 0·01 (analysis of variance).

![Graphs](image)

**FIGURE 2.** A representative profile of (a) LH, (b) testosterone and (c) inhibin in jugular (solid line) and testicular (broken line) veins 30 min before and 60 min after injection of 1 µg gonadotrophin-releasing hormone (GnRH) from a single ram. The arrows indicate the time of administration of GnRH.

(P < 0·01) and concentrations in the testicular vein were significantly (P < 0·05) higher than those in the jugular vein.

There were no significant differences in plasma concentrations of testosterone in the spermatic vein, testicular vein or testicular lymph (Table 2) but concentrations in the peripheral plasma were significantly (P < 0·05) lower than those in the testicular plasma or lymph.

**DISCUSSION**

The results of these studies show that the Leydig cells of the ram testis do not secrete inhibin in response to hCG or endogenous LH. Furthermore, it seems unlikely that the Leydig cells of the ram have significant local influences on the production of inhibin from other testicular cells, such as the Sertoli cells, since within the framework of these studies the concentrations of plasma inhibin did not change following stimulation of the function of the Leydig cells. No changes in the concentration of inhibin occurred despite marked stimulation of the secretion of testosterone from the Leydig cells by hCG and endogenous LH. These findings in the ram are in contrast to studies in man (McLachlan et al. 1988b; Fingscheidt & Nieschlag, 1989; Berger et al. 1990; McLachlan et al. 1990) and rats (Sharpe et al. 1988; Drummond et al. 1989) where administration of hCG clearly increased concentrations of inhibin as well as testosterone. The demonstration that subunits of inhibin and mRNA for the subunits of inhibin are present in the Leydig cells of rams (Risbridger et al. 1989; Roberts et al. 1989) and mice (Lee et al. 1988) suggests that the
Leydig cells in these species produce inhibin, but similar studies in the ram are lacking. It appears that species differences may exist regarding the extent to which the Leydig cells contribute to the overall circulating levels of inhibin, and in the ram the extent to which various cells in the testis produce inhibin needs to be resolved.

Although our findings suggest that the Leydig cells are not involved in the production of inhibin in the ram, the testes are clearly the major source of inhibin. This is illustrated in experiments 2 and 3 where the concentrations of inhibin in plasma from the testicular veins and from the testicular lymph were significantly higher than concentrations in peripheral plasma. Furthermore, it was found that bilateral castration of prepubertal and postpubertal ram lambs resulted in a 50% decrease in the levels of serum inhibin within 2 h of castration and a subsequent increase in serum FSH (Schanbacher, 1988). There was also a rapid fall in the levels of serum inhibin in ram lambs that were unilaterally castrated but these levels stabilized to be intermediate between those of intact rams and rams that had been bilaterally castrated (Schanbacher, 1988). Despite these findings, inhibin remained detectable in the serum of castrated rams which suggested the existence of an extratesticular source of inhibin (Schanbacher, 1988). Clarke, Tilbrook, Galloway et al. (1991) also reported that concentrations of inhibin in rams fell following castration but remained detectable over 6 days. In contrast, Lincoln & McNeilly (1989) did not detect inhibin in the plasma of castrated rams leading them to conclude that all the inhibin detected in the blood was secreted by the testes. Nonetheless, it may be possible that smaller amounts of inhibin are produced from non-gonadal sources. In an immunohistochemical study of the ram testis, Veeramachaneni et al. (1989) found staining of inhibin in the epithelia of the rete testis, ductuli efferentes, epididymis, ductus deferens, prostate, seminal vesicles and urethra and it is possible that this could have resulted from uptake of inhibin produced in the testis or non-gonadal production of inhibin. Positive immunostaining of inhibin was also observed in some non-reproductive organs of the ram including the adrenal cortex (Veeramachaneni et al. 1989) and it was found that the gene for the α subunit of inhibin was expressed in the ovine adrenal cortex (Crawford, Hammond, Evans et al. 1987). It is unknown whether these extragonadal sites at which inhibin is detected contribute to the plasma pool of inhibin.

Although the concentrations of inhibin in testicular venous blood were significantly higher than the concentrations in jugular venous blood in experiment 2, this difference was only 1.3-fold suggesting that the amounts of inhibin secreted into the blood are not large. In experiment 3 we found that the concentrations of inhibin in the testicular lymph were about five to seven times higher than in the spermatid and testicular veins and about eleven times higher than in the jugular blood. The much larger concentrations of inhibin in testicular lymph compared with the blood may indicate that inhibin is preferentially secreted into the lymph and it is possible that this may be a more important route of secretion of inhibin from the testis than in the testicular blood. Indeed, Setchell & Sharpe (1981) suggested that some testicular proteins or peptides, such as inhibin, would leave the testis in the lymph rather than the venous blood because of their molecular size, thus leading to higher concentrations in the lymph than in venous blood. The importance of the testicular lymph as a route of secretion of testicular products was illustrated when albumin was injected into the interstitial tissue of the testis of rams and boars and about 90% of the albumin leaving the testis was recovered in the lymph (Galil, Laurie, Main & Setchell, 1981). Furthermore, it was demonstrated by Scott, Burger & Quigg (1980) that ovine testicular lymph contains high levels of inhibin, and Baker, Eddie, Higginson et al. (1982) measured inhibin in the testicular lymph of rams but were unable to detect inhibin activity in the testicular venous blood. While secretion of inhibin from the testis via the testicular lymph may be an important route of secretion, it should be noted that the flow of blood through the testis is considerably greater than the flow of lymph (Setchell & Brooks, 1988), which means that relatively small amounts of inhibin in testicular venous plasma could make a major contribution to peripheral concentrations. Clearly, the extent to which inhibin in the testicular lymph contributes to peripheral concentrations can only be ascertained by considering flow rates of lymph and blood.

In addition to the secretion of inhibin into the interstitial fluid, from where it would drain into the lymphatic sinusoids and blood vessels, there is evidence that some inhibin is also secreted into the seminiferous tubule (Au, Robertson & de Kretser, 1984; Steinberger, Janeki & Jakubowiak, 1987; Sharpe, 1988). It was suggested that inhibin secreted into the lumen of the seminiferous tubule might be reabsorbed in the rete testis area because the levels of inhibin from male rats were much lower in blood sampled before (testicular venous) than after (spermatid venous) its passage through the mediastinal venous plexus (Maddocks & Sharpe, 1989; Maddocks, Hargrave, Reddie et al. 1990). These levels of inhibin in spermatid venous plasma were about two-fold higher than those in testicular venous plasma while the levels in the peripheral venous plasma and testicular venous plasma were similar (Maddocks & Sharpe, 1989; Maddocks et al. 1990).
1990). We found in experiment 3 that the differential between the concentrations of inhibin in the spermatic vein and jugular vein of rams was similar to this finding with rats but, in contrast, the concentrations in the testicular vein were significantly higher than in the jugular vein. This suggests that reabsorption of inhibin in the rete testis does not play a significant role in the ram. Nevertheless, Maddocks et al. (1990) suggested that apparent differences between species in the degree of reabsorption of inhibin in the rete testis may be due to differences in the position of the rete testis within the testis: the rat appears to be the only species investigated where it has been possible to obtain testicular blood before it passes through the region of the rete testis. In the ram, the rete testis lies along the long axis of the testis, extending for about three-quarters of the length (Sethell, 1978) making sampling of blood before the rete testis difficult.

In conclusion, the data from these experiments suggest that, in the ram, the Leydig cell does not secrete inhibin and does not significantly influence other cells within the testis to secrete inhibin. The testis is clearly the major source of inhibin in the ram and it is most likely that the major sites of production of inhibin within the testis are the Sertoli cells. Finally, the low differential in the concentrations of inhibin in testicular venous plasma and the peripheral plasma and the high concentrations of inhibin in the testicular lymph suggest that in addition to blood, the lymph may be a second major route of secretion of inhibin from the testis. Further research is necessary to confirm this view.

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