Is prolactin a gonadotrophic hormone in red deer (*Cervus elaphus*)? Pattern of expression of the prolactin receptor gene in the testis and epididymis

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ABSTRACT

This study investigated the pattern and site of expression of the prolactin receptor gene in the testis and epididymis of red deer collected during the breeding season ($n=3$). Ribonuclease protection assays using 50 µg total RNA and a 300 bp [³²P]-labelled antisense cRNA probe, generated from the extracellular domain of the red deer prolactin receptor, confirmed the expression of the receptor in both the testis and epididymis; a higher level of prolactin receptor mRNA was detected in the epididymis compared with the testis ($170.4 \pm 1.5 \times 10^3$ and $26.3 \pm 2.7 \times 10^3$ arbitrary units respectively; $P<0.05$). *In situ* hybridisation using 300 bp [³³P]-labelled sense and antisense cRNA probes generated from the extracellular domain of the receptor localised the expression sites to the seminiferous tubules and interstitial compartments of the testis and the epithelial layer of the epididymal duct. Quantification of grain numbers demonstrated a higher level of expression of the

receptor in the epididymis compared with the interstitial and seminiferous tubule compartments of the testis ($18.1 \pm 4.4 \times 10^2$, $10.1 \pm 2.0 \times 10^2$ and $8.3 \pm 0.8 \times 10^2$ grains/µm² respectively; $P<0.05$). However, no differences were detected in the level of expression of the receptor between the interstitial and seminiferous tubule compartments of the testis. Immunocytochemistry using an anti-prolactin receptor antibody, raised against a peptide sequence from the extracellular domain of the rat prolactin receptor, localised expression of the receptor gene to the Leydig cells, pachytene spermatocytes, round spermatids and elongating spermatids. In the epididymis, the receptor was localised to the epithelial layer within the epididymal ducts. Expression of the prolactin receptor gene in the red deer testis and epididymis suggests a role for the hormone in steroidogenesis and spermatogenesis. *Journal of Molecular Endocrinology* (1998) **20**, 175–182

INTRODUCTION

The red deer stag is a seasonal breeder that exhibits a circannual rhythm in reproductive activity that is inversely related to the prevailing plasma concentration of prolactin. Decreasing daylength of short days is accompanied by increased release of pituitary gonadotrophins and marked testicular recrudescence, leading to enhanced testosterone production, spermatogenesis and pronounced testicular growth (Lincoln 1971, Hochereau de Reviers & Lincoln 1978, Barrell *et al.* 1985). In contrast, as daylength becomes progressively longer, there is a decrease in gonadotrophin and testosterone

secretion and sperm production (Lincoln & Kay 1979). The secretion of prolactin from the anterior pituitary also undergoes seasonal variation in the red deer stag: long photoperiods are marked by high circulating prolactin concentrations, while low prolactin concentrations characterise short photoperiods (Curlewis 1992).

The two basic functions of the testis, spermatogenesis and steroidogenesis, are controlled by follicle-stimulating hormone and luteinising hormone (LH) respectively (Amann & Schanbacher 1983). However, recently, there has been a growing interest in the positive effect that prolactin may have on testicular function. In rams, a positive

correlation has been described between prolactin and testicular growth, testosterone production and LH receptor expression (Regisford & Katz 1993, 1994). In addition, Lincoln *et al.* (1996) have demonstrated a low-amplitude cycle in testicular diameter in hypothalamo-pituitary disconnected (HPD) Soay sheep. In HPD rams, the testicular cycle persists despite the absence of gonadotrophin secretion in these rams and is positively correlated with prolactin concentration. These data strongly support the notion that prolactin acts directly at the testicular level and acts synergistically with gonadotrophins to regulate long-term cycles in testicular activity in seasonally breeding mammals such as the red deer and sheep. The increase in prolactin concentrations during long photoperiods is believed to be essential for priming the testis for redevelopment during the short-day period, at which time there is increased secretion of gonadotrophins (Sanford & Dickson 1980, Howles *et al.* 1982, Sanford *et al.* 1984a, b).

We have previously reported the cloning and sequencing of the prolactin receptor cDNA in red deer, which is predicted to encode a protein of 557 amino acids. The sequence in red deer differs from the long form of most other species by a 3' truncation of the cytoplasmic domain. Despite this deletion, the receptor retains its functionality as assessed by tyrosine phosphorylation of the receptor, the associated protein Jak2 and the induction of transcription of the prolactin-responsive β -casein promoter (Jabbour *et al.* 1996). In northern hybridisation studies, prolactin receptor expression was evident in the testis of red deer stags during both the breeding and non-breeding seasons (Clarke *et al.* 1995). In addition, previous studies have confirmed that, in the testis, two forms are expressed that are encoded by transcripts of approximately 3.5 and 1.7 kb (Clarke *et al.* 1995, Jabbour *et al.* 1996). In the present study, we investigated whether, in red deer, prolactin exerts a direct effect on reproductive function through interaction with its membrane-bound receptors in the testis and epididymis. To this end, expression of the prolactin receptor gene was ascertained in the red deer testis and epididymis during the breeding season using the ribonuclease protection assay (RPA) technique. In addition, the site of expression of the receptors in both tissues was localised by *in situ* hybridisation using sense and antisense cRNA probes generated from the extracellular domain of the receptor. Finally, the prolactin-receptor-expressing cells in the testis and epididymis were identified by immunocytochemistry using an antiprolactin receptor antibody.

MATERIALS AND METHODS

Animal and tissues

Testis and epididymis were collected from three mature and sexually active red deer stags in September/October; this coincides with the period of transition from the non-breeding to the breeding season in this species in the United Kingdom. The testis and epididymis were removed shortly after the animals were killed. Samples utilised for RNA extraction were immediately snap frozen in liquid nitrogen and stored at -70°C until required for analysis. Tissues used for *in situ* hybridisation were mounted in tissuetek, snap frozen in isopentane in liquid nitrogen and stored at -70°C until required for further use. Samples utilised for immunocytochemistry were fixed in Bouin's solution for 5 h and stored in 70% ethanol overnight before processing for embedding in paraffin wax.

Preparation of the prolactin receptor cDNA construct

For preparation of the prolactin receptor riboprobes, a 300 bp fragment (between bases -105 and 195 from the start codon) from the cervine prolactin receptor sequence was amplified by PCR using a clone encoding the full prolactin receptor cDNA (Jabbour *et al.* 1996) and the following primers: forward $5'$ -GCTAAAGAACGCTTCTGTTG- $3'$ and reverse $5'$ -GTGGTAAGTCAGCGTG- $3'$. The amplified PCR product was subcloned into the pGEM vector and its identity confirmed by sequencing using the dideoxy nucleotide chain-termination method (Sanger *et al.* 1977). This construct was used for generation of riboprobe transcripts used in the RPA and *in situ* hybridisation studies.

RNA extraction and ribonuclease protection assay

RNA was extracted from the testis and epididymis using the guanidinium thiocyanate method (Chomczynski & Sacchi 1987) and RNA yields were estimated by spectrophotometry at 260 nm. In addition, liver RNA was prepared using the same procedure and utilised in the RPA as a positive control. Liver tissue has been shown previously to express the prolactin receptor gene abundantly in red deer at this stage of the breeding season (Clarke *et al.* 1995).

For the RPA, an antisense cRNA was prepared from plasmid containing a 300 bp prolactin receptor cDNA (described above) after linearisation with SacI. The RPA was conducted using the Ambion RPA II kit (AMS Biotechnology Europe, Witney,

Oxon, UK) as instructed by the manufacturer. Briefly, the linearised plasmid was incubated with T7 RNA polymerase in the presence of [32 P]UTP (800 Ci/mmol, Amersham International plc, Amersham, Bucks, UK) mixed with loading dye (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 30% glycerol, 0.5 mM EDTA, 0.025% SDS) heated at 95 °C for 3 min and separated on a denaturing 5% (w/v) acrylamide gel for 3 h at 50 W. The gel was then covered in Saran wrap and exposed for 2 min to XAR-5 film (Kodak, IBI, Cambridge, UK) to determine the location of the full-length radiolabelled cRNA. The appropriate portion of the gel was cut out, placed in an Eppendorf tube together with 350 μ l elution buffer (containing 0.5 M ammonium acetate, 1 mM EDTA and 0.2% w/v SDS) and incubated overnight at 37 °C. The activity of 2 $\times$ 1 μ l aliquots was determined by liquid scintillation spectroscopy. The average of the samples was determined and the volume of radiolabelled probe required to give 2 $\times$ 10⁵ c.p.m. calculated. Total RNA (50 μ g) from testis ($n=3$) epididymis ($n=3$), liver ($n=1$; used as a positive control) and yeast ($n=2$; used as reaction controls in the presence or absence of RNase digestion to establish the specificity of the hybridisation reaction and the size of the unprotected RNA fragment) was mixed with the radiolabelled probe and hybridisation buffer, heated to 90 °C for 4 min and incubated overnight at 45 °C. The next day, single-stranded RNAs were digested using 250 units/ml RNase A and 10 000 units/ml RNase T1 at 37 °C for 30 min. The protected RNA was precipitated and resuspended in gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA and 0.025% SDS), heated at 95 °C for 3 min and separated on a 5% acrylamide gel under denaturing conditions. The experiment was repeated twice and the gels were dried under vacuum and initially exposed to a phosphorescent screen (Molecular Dynamic, Chesham, Bucks, UK) followed by an autoradiographic film (XAR-5, Kodak).

Integrity of RNA and the relative amount of total RNA in each reaction was determined by including radiolabelled cRNA prepared from an 18S ribosomal standard cDNA in each reaction. The amounts of prolactin receptor RNA and 18S ribosomal RNA were counted on a phosphorimager (Molecular Dynamics). Differences between the amounts of prolactin receptor mRNA in the testis and epididymis were evaluated using analysis of variance.

***In situ* hybridisation**

Cryostat tissue sections (5 μ m thick) were thaw-mounted onto 3-aminopropyltriethoxy silane

(TESPA)-coated and baked slides (2% TESPA in acetone; Sigma Chemical Co., Poole, Dorset, UK) and fixed for 5 min in 4% (wt/vol) paraformaldehyde made up in 0.1 M PBS. The slides were acetylated and prehybridised for 2 h at 55 °C in prehybridisation buffer consisting of 50% deionised formamide, 5 $\times$ SSPE (1 $\times$ SSPE contains 0.15 M NaCl, 10 mM NaH₂PO₄·H₂O and 1 mM EDTA), 5 $\times$ Denhardt's solution, 200 μ g/ml yeast transfer RNA, 200 μ g/ml denatured salmon sperm DNA and 1% SDS. Hybridisation was then performed overnight in hybridisation buffer (prehybridisation buffer plus 4% dextran sulphate and 10 mM dithiothreitol) containing 1 $\times$ 10⁶ c.p.m. 32 P-labelled cRNA. Excess probe was removed by washing in 4 $\times$ SSC (1 $\times$ SSC contains 0.15 M NaCl, 15 mM sodium citrate, pH 7) at room temperature before the sections were treated with ribonuclease A (20 μ g/ml). The sections were subsequently incubated with 4 $\times$ SSC, 2 $\times$ SSC and 0.1 $\times$ SSC for 30 min each at room temperature. Tissues were dehydrated progressively in alcohols (50%, 85% and 95%) containing 0.3 M ammonium acetate and air-dried before they were dipped in NTB 2 emulsion (Kodak). After incubation in a humidified box overnight, tissues were placed in a sealed dark box at 4 °C for 3 weeks, developed with D19 developer, and fixed with Unifix (Kodak) at 14 °C in the dark-room.

Labelled sense and antisense cRNA were synthesised by incubation of linearised template (1 μ g) with 50 μ Ci [32 P]UTP (2000 Ci/mmol; Amersham) in the presence of T7 or SP6 RNA polymerase for 30 min at 37 °C according to the manufacturer's recommendations (Promega, Southampton, UK).

Population density of grains in sections of testis and epididymis utilised in the *in situ* hybridisation studies were estimated using an Image Pro image analysis software linked to an Olympus BH2 microscope fitted with a dark-field condenser. The population density of grains determined for each section was the mean of grain density determined in a total of 25 fields of view that were selected in a systematic manner from a random start; this was established for the sense and antisense treatments in the testis and epididymis. The data were analysed by analysis of variance and pairwise comparisons were conducted using Fisher's protected LSD.

Histology and immunocytochemistry

Sections 5 μ m thick were cut and mounted on slides coated with 2% TESPA in acetone. Slides were then dried overnight at 50 °C before dewaxing in histoclear. Tissues were rehydrated in graded ethanol and washed in water followed by

Tris-buffered saline (TBS: 0.05 M Tris-HCl pH 7.4, 0.85% NaCl). Sections were treated with 10% hydrogen peroxide in methanol for 30 min and then blocked for 30 min with normal swine serum (NSS) diluted 1 : 5 in TBS+5% BSA. The primary antibody for the prolactin receptor (kindly donated by Dr P M Ingleton, School of Medicine, University of Sheffield) was raised against a 16 amino acid synthetic peptide corresponding to residues 53–68 of the external domain of the rat prolactin receptor (Nevalainen *et al.* 1996). This region shares an 87.5% homology with the red deer prolactin receptor amino acid sequence. The polyclonal antibody was diluted in NSS-TBS+5% BSA (see above) and incubated on the sections overnight at 4 °C under plastic coverslips. Control sections were incubated with non-immune rabbit serum. After removal of coverslips, sections were washed twice in TBS (5 min each), incubated for 30 min with biotinylated swine anti-rabbit immunoglobulin (Dako, High Wycombe, Bucks, UK) diluted 1 : 500 in NSS-TBS, then washed again twice in TBS (5 min each) and incubated with peroxidase antiperoxidase conjugated to avidin-biotin complex (Dako) for 30 min at room temperature. Colour reaction was developed by incubation in a mixture of 0.05% 3,3'-diaminobenzidine (Sigma) in 10 ml 0.05 M Tris-HCl buffer (pH 7.4) and 0.033% hydrogen peroxide.

RESULTS

The results from a typical ribonuclease protection assay are shown in Fig. 1. The ribonuclease protection assays confirmed the expression of the prolactin receptor gene in both the testis and the epididymis of the red deer at the beginning of the breeding season (Fig. 1); no protected bands were observed in the yeast RNA sample after RNase digestion. Whereas all samples contained comparable levels of 18S ribosomal RNA, the amount of prolactin receptor RNA was significantly greater in the epididymis than in the testis: the arbitrary counts assigned ($n=3$) using a phosphorimager were $170.4 \pm 1.5 \times 10^3$ and $26.3 \pm 2.7 \times 10^3$ respectively ($P<0.05$; mean $\pm$ S.E.M.).

The results from a typical *in situ* hybridisation of the testis and epididymis are presented in Fig. 2; this pattern of expression was detected in each animal. The sections confirm the expression of the receptor gene both in the Leydig cells and within the seminiferous tubule compartment of the testis. In addition, in the epididymis the expression was localised to the epithelial layer of the epididymal duct. In order to compare the level of expression of the receptor gene in the epididymis and the testis,

FIGURE 1. RPA conducted using 50 µg total RNA isolated from testis and epididymis of red deer and a 300 bp homologous prolactin receptor cRNA probe generated from the extracellular domain. Liver RNA (50 µg) was included as a positive control and yeast RNA with (+) and without (–) RNase digestion were used as reaction controls. Integrity of RNA and the relative amount of total RNA in each reaction was determined using a ribosomal 18S cRNA probe.

grain counts were performed on all sections. The population density counts of grains (mean $\pm$ S.E.M.) are illustrated in Fig. 3; the grain counts in the sections exposed to the sense probe were negligible and comparable across all sections. Hybridisation with the antisense probe demonstrated a comparable level of expression of the receptor gene between the Leydig cells and the seminiferous tubule compartment of the testis ($10.1 \pm 2.0 \times 10^2$ and $8.3 \pm 0.8 \times 10^2$ grains/µm² respectively; $n=3$), but expression of the receptor gene in the epididymis ($18.1 \pm 4.4 \times 10^2$ grains/µm²; $n=3$) was significantly greater ($P<0.05$) than that observed in either compartment of the testis.

Immunohistochemistry using an antibody that was directed against a conserved peptide sequence derived from the extracellular domain of the rat prolactin receptor localised further the site of expression of the receptor gene in the red deer testis and epididymis (Fig. 4). In the testis, expression was localised to the Leydig cells within the interstitial region. In addition, in the seminiferous tubules localised expression of the receptor was detected in pachytene spermatocytes, round spermatids and elongating spermatids. There was no notable expression localised in mature spermatozoa. In the epididymis, immunohistochemistry localised the site of expression of the receptor gene to the epithelial cell layer.

DISCUSSION

The data presented here confirm the expression of the prolactin receptor gene in the testis and epididymis of red deer. This suggests that, in red deer, the pituitary hormone, prolactin, may be involved in testicular function. Localisation of expression of the receptor gene in the seminiferous tubule (germ cells) and interstitial (Leydig cells)

FIGURE 2. *In situ* hybridisation of prolactin receptor in the testis and epididymis of red deer. Prolactin receptor expression was localised in Leydig cells (L), and the seminiferous tubular (ST) compartment of the testis (A, B: dark- and light-field images after hybridisation with the antisense probe; C: dark-field image after hybridisation with the sense probe) and in the epithelial layer within the epididymis (D, E: dark- and light-field images after hybridisation with the antisense probe; F: dark-field image after hybridisation with the sense probe). Bar represents 100 μ m.

compartments implicates prolactin as a possible gonadotrophic hormone regulating the processes of both spermatogenesis and steroidogenesis in ungulate species. In addition, the expression of prolactin receptors in the epididymis of red deer suggests a more generalised function for the hormone in male reproductive physiology.

Expression of the prolactin receptor gene in the Leydig cells is consistent with the well-described role of prolactin in steroidogenesis. Normal physiological prolactin concentrations have been shown to be important for maintenance of testosterone secretion during the breeding season in sheep (Yarney & Sanford 1989). Also in sheep, treatment with bromocriptine during the autumn months to suppress prolactin secretion results in reduced testosterone secretion (Regisford & Katz 1993). Evidence in rodent species suggests that prolactin influences testosterone secretion through

the stimulation and maintenance of expression of LH receptors in Leydig cells (Klemcke *et al.* 1984, Takase *et al.* 1990) or by regulating specific enzymatic steps in androgen biosynthesis (Chandrashekar & Bartke 1988).

Expression of prolactin receptors within the seminiferous tubules suggests that prolactin may be involved directly in the process of spermatogenesis. There are considerable data supporting a positive relationship between prolactin secretion and testicular physiology in ungulate species. In intact rams, treatment with bromocriptine to suppress circulating prolactin concentrations during the sexually inactive period causes a decrease in testicular size (Sanford & Dickson 1980, Regisford & Katz 1993) and spermatozoa production (Sanford & Dickson 1980), or a delay in testicular recrudescence. Circannual cycles in testicular activity were better expressed in rams maintained under long days,

FIGURE 3. Population density of grains in sections of testis (the seminiferous tubule compartment and Leydig cells) and epididymis used in the *in situ* hybridisation studies for the prolactin receptor. Values are mean $\pm$ S.E.M. of grain density determined in a total of 25 fields of view that were selected in a random and systematic manner. Open bars, sense probe; solid bars, antisense probe.

when circulating prolactin concentrations varied from high to low with a fixed temporal association with testicular volume, compared with rams living continuously under short days, when prolactin secretion was chronically suppressed (Howles *et al.* 1982).

Further evidence for a stimulatory role for prolactin in testicular activity has been established in HPD Soay rams. These rams express a persistent low-amplitude cycle in testicular diameter that is positively correlated with the long-term changes in prolactin secretion (Lincoln *et al.* 1996). Morphometric analysis of the testis of HPD rams demonstrated evidence of spermatogenesis, with the presence of pachytene/diplotene primary spermatocytes, which may be regulated by prolactin (Lincoln *et al.* 1996). These data suggest that, during the summer months, prolactin may be involved in the initiation of the process of spermatogenesis, and that it facilitates the rapid reactivation of testicular function in response to the increased secretion of gonadotrophins, and consequently androgens, in the autumn months of the breeding season. This hypothesis is supported by the detection of prolactin receptor gene expression during the breeding and non-breeding seasons of red deer (Clarke *et al.* 1995). Moreover, localisation of expression of the prolactin receptor gene in the seminiferous tubule compartment and in Leydig

cells suggests a role for prolactin in the processes of spermatogenesis and steroidogenesis in the red deer testis. The pattern of expression of the prolactin receptor gene on germ cells may imply a role for prolactin in the differentiation of spermatocytes or the regulation of the cell division cycle, or both, by acting as a meiotic inducer. In concert with gonadotrophins, prolactin may be an important factor in enhancing the efficiency of spermatogenesis. This is supported further by observations that administration of prolactin to immature hypophysectomised rats increases the number of spermatocytes (Dombrowicz *et al.* 1992) that are known to express the receptor *in vivo* (Hondo *et al.* 1995).

Prolactin is not able to enter the adluminal compartment of the seminiferous epithelium because of the blood-tubule barrier. This raises the question of the mechanism of transport of prolactin across such a barrier. One possibility is that it occurs through the expression of prolactin transport proteins that would act as prolactin transmembrane carriers, to ensure transport of prolactin from the interstitial into the seminiferous tubule compartment. In red deer testis, two forms of the prolactin receptor have been identified which are encoded by transcripts of approximately 3.5 and 1.7 kb. The 3.5 kb transcript is known to encode the long-form membrane-bound receptor (Jabbour *et al.* 1996), but the smaller transcript may encode a non-membrane-bound receptor protein that would act as a prolactin transporter (Jabbour & Kelly 1997). Future work will investigate the function of the shorter prolactin receptor transcript and the possible differential site of expression of the two prolactin receptor isoforms in the testis. This may provide further insight into the exact role of prolactin in spermatogenesis and its possible mode of transport into the seminiferous tubule compartment.

Prolactin receptor expression was also detected, by *in situ* hybridisation and immunocytochemistry, in the epithelial layer of the epididymal duct. The exact role of prolactin in the epididymis remains to be elucidated. However, prolactin is believed to maintain epididymal weight, stimulate testosterone uptake, promote fluid exchange across the epididymal epithelium (Brumlow & Adams 1990) and stimulate the production of epididymal steroids that contribute to the steroid content of seminal plasma (Hamilton & Fawcett 1970). It is also possible that prolactin is involved in promoting mitogenesis and regeneration of the epithelial layer of the epididymis. Lincoln (1971) has shown that the epididymal weight and the height of the epithelial layer undergo seasonal variation, with initiation of growth coinciding with the period of increased

FIGURE 4. Immunocytochemical staining for prolactin receptor in the testis (A–D) and epididymis (E) of red deer. In the testis, expression of the receptor was localised in pachytene spermatocytes (P), round (R) and elongating (E) spermatids and Leydig cells (L). In the epididymis, expression of the receptor was localised to the epithelial layer (EL). Negative controls for testis (F) and epididymis (G) are shown. The sections were counterstained with haematoxylin. Bar represents 100 μ m.

endogenous prolactin secretion. Prolactin has been demonstrated to promote cell division and mitogenesis after interaction with its membrane-bound receptors (Das & Vonderhaar 1995). This function of prolactin is believed to be mediated through activation of the mitogen activated protein kinase pathway (Das & Vonderhaar 1995).

In conclusion, this study has confirmed the expression of prolactin receptors in the red deer testis and epididymis. Localisation of the site of expression of the receptors to the seminiferous tubule and interstitial compartments of the testis suggests that prolactin may promote both the spermatogenic and the steroidogenic function of the

testis, and thus act as a gonadotrophic hormone in seasonally breeding mammals such as red deer.

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