Introduction

LH and FSH stimulate ovarian cells by specific binding to their membrane receptors. In the ovary, LH receptors (LHR) are found in theca and granulosa cells of the preovulatory follicle, in the corpus luteum and, in some species such as rats, in interstitial cells (Zeleznick et al., 1974; McFarland et al., 1989). The transcripts of LHR have been shown to undergo extensive alternative splicing. Several splice variants for LHR have been described, at least 11 in rats (McFarland et al., 1989; Segaloff et al., 1990; Aatsinki et al., 1992), four in pigs (Loosfelt et al., 1989) and sheep (Bacich et al., 1994) and two in humans (Frazier et al., 1990; Minegishi et al., 1990). However, it remains unknown whether any of these splice variants are translated in gonadal tissues.

During seasonal anoestrus, most breeds of sheep are anovulatory, although the ovaries of anoestrous ewes are relatively active with respect to follicular development and steroidogenesis (Bartlewski et al., 1998). The population of antral follicles is similar to that found during the breeding season, with no change in the total number of antral (McNatty et al., 1984) or ovulatory sized follicles (Cahill, 1981), and the largest ovarian follicles exhibit a wave-like growth pattern. Reduced LH secretion and ovarian responsiveness to gonadotrophic stimuli, especially LH, may be the major cause of anovulation during anoestrus (Legan et al., 1979; McNatty et al., 1981, 1984). The aim of the present study was to determine whether, in ewes, the changes from the ovulatory to the anovulatory state, which are accompanied by a decrease in LH tonic secretion and in ovarian response to LH (Legan et al., 1985), may be correlated with changes in the regulation of LH receptor expression by alternative splicing of its mRNAs. For this purpose, splice variants of mRNAs encoding LH receptor were identified using RT–PCR and splicing was compared in ovarian follicles during the oestrous and anoestrous seasons.

Materials and Methods

Animals

Adult cyclic (n = 6) and non-cyclic (n = 6) ewes were used for this study. Non-cyclic ewes were treated with progestagen (intravaginal fluorogestone acetate sponges, 40 mg; Intervet, Angers) for 15 days to synchronize oestrus, injected with 500 iu eCG as standard (Intervet) and killed 24 h after sponge removal. Cyclic ewes were synchronized with progestagen sponges, either injected (n = 3) or not (n = 3) with eCG, and killed 36 h after sponge removal (that
is, in the follicular phase of the cycle). Ovaries were placed in medium B₂ (Menezo, 1976) and transported to the laboratory.

Separation of granulosa and theca cells

Individual follicles (2.5–6.0 mm) obtained from three eCG-treated cyclic ewes and three eCG-treated non-cyclic ewes were dissected and measured using a millimetre scale. For each follicle, the antral fluid was removed by puncture, and the collapsed follicle was placed open in medium B₂. Granulosa cells were isolated by scraping the interior surface of the follicle wall with a platinum loop. Smears of granulosa cells were placed on histological slides, fixed in methanol:formaldehyde:acetic acid (80:15:5) and stained with Feulgen stain for quality determination under a microscope. The purity of theca cells was challenged by RT–PCR using specific FSH receptor primers to eliminate cross-contaminations, and only FSH receptor-negative samples were included in this study. Follicles were classified on the basis of morphology as healthy (frequent mitosis and no, or few, pyknotic bodies in the granulosa cells) or atretic. Atretic follicles were not submitted to further analysis and were withdrawn from this study. Granulosa and theca cells of each individual follicle were then frozen separately on dry ice and stored at –80°C until RNA extraction.

RT–PCR assays

Total RNA was extracted from the granulosa and theca cells of individual healthy follicles using RNAXEL (Eurobio, les Ulis) and final RNA concentrations were determined by measuring absorbance using a spectrophotometer. RNA was preheated for 10 min at 65°C, immediately cooled on ice and reverse transcribed for 50 min at 42°C in 20 μl reaction mixture containing RT buffer (0.25 mol Tris–HCl l⁻¹, pH 8.3, 0.375 mol KCl l⁻¹ and 15 mmol MgCl₂ l⁻¹), 500 μmol deoxy-NTPs l⁻¹ (Ultrapure solution; Pharmacia, LKB, Uppsala), 200 μU Mu-MLV (murine Moloney leukaemia virus) reverse transcriptase (Gibco BRL, Cergy Pontoise) and 17.5 U RNase inhibitor (Pharmacia). The RT reaction was heated for 5 min at 95°C. PCR reactions contained cDNA (2 μl), 1 mmol deoxy-NTPs l⁻¹, 1 mmol l⁻¹ of each specific primer and 1 U Taq polymerase (Appligène, Illkirch). Amplification primers were deduced from ovine LHR published sequences (GenBank accession numbers L36329 and L36115, respectively). The ovine LH receptor OLHR3–OLHR8 primers define LHR A, an extracellular domain fragment of 351 bp, whereas the OLHR9–OLHR11 pair define LHR B, a 437 bp fragment situated before the membrane domain (Fig. 1). The primer sequences for the LHR A region were 5’-TCCCTGGAAGATAGACTCATTCGC-3’ (OLHR3) and 5’-TCTTCTCGGCGTGCATTTC-3’ (OLHR8). The primer sequences for the LHR B region were 5’-AACCTGCCAACAAAAGAG-3’ (OLHR9) and 5’-ATGGTTATAATGCGCC-3’ (OLHR11). The reaction mixture was subjected to PCR amplification in a thermal cycler. Each cycle consisted of denaturation for 1 min at 95°C, annealing for 1 min at 63, 53 and 55°C, for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LHR A, LHR B, respectively. PCR products were analysed on 2% ethidium bromide agarose gels.

Semiquantitative analysis

Semiquantitative RT–PCR was carried out using the housekeeping gene GAPDH as an internal standard. GAPDH is expressed constitutively in both granulosa and theca cells and has already been used as a reference in several studies on the regulation of gonadotrophin receptor gene expression in ovarian cells (Smith et al., 1996; Mamluck et al., 1998). In the present study, the semiquantitative RT–PCR method developed by Murphy et al. (1990) was used to examine the amount of expression of different forms of transcripts of mRNA encoding LHR. For each sample, mRNA encoding GAPDH was amplified and all values for cDNA encoding LH receptor were expressed relative to GAPDH. The number of cycles was varied to determine the optimal number that would allow detection of the appropriate messages, while retaining amplification for these genes in the exponential phase (Fig. 2). Oligonucleotide primers used for GAPDH PCR amplification were 5’-ACTGGAAGATGGC-3’ and 5’-CAGTGGT-
TCACGCCCATCACA-3’. The PCR conditions were 1 min at 94°C for denaturation, followed by 1 min at 62°C and 1 min at 72°C.

**Southern blotting**

For Southern hybridization of PCR products (LHR), fragments generated by RT–PCR were denatured in a buffer containing 0.2 mol NaOH l⁻¹ and 0.5 mol NaCl l⁻¹, neutralized in a buffer containing 0.5 mol Tris l⁻¹, pH 8, and 0.5 mol NaCl l⁻¹ and blotted from agarose onto nitrocellulose membranes (Hybond-C; Amersham Life Science, Orsay) in 10 × SSC (1 × SSC was 150 mmol NaCl l⁻¹, 15 mmol sodium citrate l⁻¹, pH 7). Fixation was carried out by cross-linking with UV light for 2 min. Prehybridization was performed for 4 h at 65°C in a hybridization buffer containing 3.6 mol NaCl l⁻¹, 0.4 mol Na₂HPO₄ l⁻¹, 0.4 mol NaH₂PO₄ l⁻¹, 0.5 mol EDTA l⁻¹, 1% (w/v) SDS (Prolabo, Fontenay-sous-bois), 6% (w/v) polyethyleneglycol 6000 (Prolabo) and 5% (w/v) low fat milk (Regilait, Lyon). Hybridization was performed overnight at 65°C after the addition of 32P-labelled internal cDNA probes (extending from exon 3 to exon 7 for LHRA products, or exon 10 to exon 11 for LHRB products). Blots were washed two times at 42°C in 4 × SSC, 0.1% SDS, dried and then exposed to Kodak X-ray film.

**Sequencing**

The RT–PCR products for LHR were subjected to electrophoresis through 2% NuSieve agarose gel (Q-Biogene, Illkirch). Bands were dissected out on a UV transilluminator,
and DNA was extracted for direct automatic sequencing (ABI, Roissy).

**Statistical analysis**

For statistical analysis, follicles were classified according to size: small (2.5–3.5 mm in diameter), medium (4.0–5.5 mm in diameter) and large follicles (6.0–6.5 mm in diameter). Two-way ANOVA was used to determine the statistical effect of follicular size or season of reproduction on the amounts of LHR mRNA splice variants. Results were further analysed by Student’s t test using the residual variance from the initial ANOVA. Data are represented as means ± SEM and a value of $P < 0.05$ was considered significant.

**Results**

**Semiquantitative analysis of the different RNA transcripts**

Expression of the different variants of mRNA transcripts encoding LHR in theca cells of cyclic and non-cyclic ewes were semiquantified. Initially, the optimal number of PCR cycles for LH receptor and GAPDH cDNA was determined (Fig. 2). The intensities of all bands increased linearly when the amounts of total RNA increased from 100 to 500 ng per tube (Fig. 2a). The intensities of bands for GAPDH, LHR1 and LHR3 increased from cycle 19 to cycle 25 and reached a plateau after 25 cycles, whereas the LHR2 product was in its exponential phase of amplification from cycle 19 to cycle 27 (Fig. 2b). Accordingly, all subsequent RT–PCR analysis was performed using amounts of 400 ng for RT followed by 25 amplification cycles during PCR.

**LH receptor mRNAs detected by RT–PCR in ovarian tissues**

Primers OLHR3 and OLHR8 were used to amplify a part of the extracellular domain of the ovine LHR (LHRA), and a single band of amplified DNA was observed after RT–PCR in both the granulosa and theca cells of individual follicles collected from cyclic and non-cyclic ewes. No bands of LHRA were apparent after RT–PCR of granulosa cells of small follicles of 2 and 3 mm in diameter under the conditions of the study (Fig. 3). The nucleic acid sequence of the LHRA product (351 bp) was determined (sequence not shown) and corresponds to the sequence published in GenBank.

In theca cells from oestrous ewes, RT–PCR with OLHR9–OLHR11 primers amplified mostly the full-length LHR1, although in RT–PCR of large follicles, a small amount of LHR2 can also be observed. In contrast, when RNA was extracted from the theca cells of individual follicles collected from non-cyclic ewes, a different pattern of transcripts was observed and LHR1, LHR2 and LHR3 were observed in RT–PCR of small follicles. Moreover, the amounts of the two alternatively spliced species, LHR2 and LHR3, increased with follicle size in theca cells from anoestrous ewes (Fig. 4a). In RT–PCR of granulosa cells (Fig.
4b), a constant pattern of transcripts was observed, whether follicles were obtained from oestrous or anoestrous ewes. In granulosa cells, the alternative transcript LHR3 was more abundant than LHR1 and LHR2; abundance of LHR1 was similar to that of LHR2. Expression of mRNA encoding GAPDH in theca cells of follicles did not vary according to breeding season (Fig. 4c).

Southern hybridization with an LHR probe was used to confirm the differential expression pattern of spliced variants in theca cells from anoestrous (presence of LHR1, LHR2 and LHR3) and oestrous (absence of LHR3) ewes. Moreover, hybridization of PCR products eliminated the smallest transcript of about 100 bp that appeared in some lanes on ethidium bromide-stained gels as a non-specific PCR contaminant (data not shown). The sequence of the largest product (LHR1, 437 bp) is identical to the ovine LH receptor recorded in GenBank (Fig. 5). The LHR2 form corresponds to a deletion of exon 10 by fusion, in frame, between exons 9 and 11. The LHR3 form (172 bp) corresponds to a splicing between the 3'-end of exon 10 and an acceptor site within the coding region of exon 11 which generates a frameshift.

**Fig. 5.** cDNA sequences of LH receptor (LHR) splice variants, LHR2 and LHR3, amplified with ovine LH receptor OLHR9–OLHR11 primers, using RNA from the theca cells of follicles. The two vertical bars indicate junction of exons 9 and 10 and 10 and 11, respectively. The arrow represents the 3'-splice site in exon 11 for the LHR3 splice variant.

Irrespective of follicular size, expression of mRNA encoding GAPDH in theca cells of follicles did not vary according to breeding season (Fig. 4c).

Southern hybridization with an LHR probe was used to confirm the differential expression pattern of spliced variants in theca cells from anoestrous (presence of LHR1, LHR2 and LHR3) and oestrous (absence of LHR3) ewes. Moreover, hybridization of PCR products eliminated the smallest transcript of about 100 bp that appeared in some lanes on ethidium bromide-stained gels as a non-specific PCR contaminant (data not shown). The sequence of the largest product (LHR1, 437 bp) is identical to the ovine LH receptor recorded in GenBank (Fig. 5). The LHR2 form corresponds to a deletion of exon 10 by fusion, in frame, between exons 9 and 11. The LHR3 form (172 bp) corresponds to a splicing between the 3'-end of exon 10 and an acceptor site within the coding region of exon 11 which generates a frameshift.

**Changes in expression of LHR mRNA in theca cells according to season and follicular size**

Irrespective of follicular size, expression of mRNA encoding LHR3 in theca cells varies with the annual cycle of reproduction (Fig. 5). LHR3 mRNA expression was more abundant in the theca cells of follicles collected during...
anoestrus than in those collected during oestrus (1.6 ± 0.2, \( n = 20 \) versus 0.19 ± 0.06, \( n = 22 \); arbitrary units; \( P < 0.001 \)). During oestrus, thecal expression of LHR3 mRNA was only just detectable in the three categories of follicle (small: 0.19 ± 0.06, \( n = 8 \); medium: 0.2 ± 0.04, \( n = 9 \); and large: 0.18 ± 0.04, \( n = 5 \)). In contrast, during anoestrus, LHR3 mRNA was highly expressed in small, medium and large follicles (1.3 ± 0.1, \( n = 8 \); 1.8 ± 0.2, \( n = 8 \); and 1.7 ± 0.3, \( n = 4 \), respectively) and this expression was 2.1-fold higher (\( P < 0.01 \)) than the corresponding expression of LHR1 and LHR2 mRNA. During anoestrus, LHR3 mRNA expression was greater (\( P < 0.05 \)) in the theca cells of medium and large follicles than it was in small follicles, whereas there were no differences in the relative intensity of the LHR2 and LHR3 forms according to size.

**Discussion**

Several transcripts for LH receptor (ranging from 1.2 kb up to 7.5 kb) have been demonstrated to occur in the gonads of pigs (Loosfelt et al., 1989), rats (McFarland et al., 1989; Wang et al., 1991), humans (Minegish et al., 1990) and rabbits (Laborde et al., 1996). The origin of the variety of LH receptor transcripts has been attributed to alternative transcription start sites, alternative RNA splicing, or the use of alternative polyadenylation site heterogeneity within their 3’-untranslated regions.

The splicing of LHR3 was identical to that of the B splice variant found in rats (Bernard et al., 1990; Segaloff et al., 1990), pigs (Loosfelt et al., 1989), sheep (Bacich et al., 1994, 1999) and cows (Kawate and Okuda, 1998). The present study demonstrated that, in the theca cells of follicles collected during anoestrus, the LHR3 splice variant was twofold as abundant than full-length LHR1. In the ovine ovary, Bacich et al. (1994) reported that the B splice form is 5.0–3.5-fold more abundant than the full-length A form. In the pig testis, < 60% of the LHR transcripts are of the A form (Loosfelt et al., 1989) and, in the rat corpus luteum, < 50% are of the A form, and > 50% are of the B form (Bernard et al., 1990). Thus, the B splice variant (LHR3 in the present study) is a major form of the mRNA encoding LHR. Splicing requires conserved sites at both the 5’- and 3’-ends of the spliced region and at a further site at least 20 bases upstream of the 3’-acceptor site. The LHR3 form splices to a 3’-acceptor site within exon 11 of the coding region of the full-length LHR1 form. The sequence at this site is UUUGCAG/A (where / = splicing site) and differs slightly from the consensus 3’-acceptor site (YnNYAG/C; Y = C or U, N = U, A, C or G) (Smith et al., 1989).

A truncated form of LHR mRNA, encoding only the extracellular domain, is present in fetal rat ovaries and testes, and the expression of the full-length receptor mRNA occurs simultaneously with the appearance of the functional receptor both at the onset of ovarian development at 7 days after birth (Sokka et al., 1992) and upon maturation of testicular Leydig cells (Zhang et al., 1994).
Studies in situ indicate that these truncated LH receptor mRNAs are expressed uniformly by differentiating theca, granulosa and interstitial cells in rat ovary (Sokka et al., 1996). Each of these types of cell will eventually express the full-length receptor, indicating that the number of receptors is regulated by splicing of the primary transcript.

During anoestrus, the pattern of follicle development is similar to that found during the breeding season (Cahill, 1981) and the ovaries of anoestrous ewes are relatively active. Insufficient tonic LH secretion and a decrease in ovarian response to LH may be the major causes of anovulation during anoestrus (Legan et al., 1985). Moreover, LH promotes follicle and oocyte maturation in mice through theca cell, but not granulosa cell, receptors (Cortvrindt et al., 1998). This finding indicates that regulation of alternative splicing during oestrus and anoestrus is under the control of changes in tonic LH secretion or metabolic signals. Regulation of alternative splicing events by hormone- and tissue-specific factors in numerous genes is well documented (for a review, see Chew, 1997). The effect of such factors may favour certain splice sites located upstream or downstream of exon 11, leading either to the long form of LH receptor (during oestrus) or to its splice variants (during anoestrus). Regulation of the selection of intron splice sites implicates factors such as the U1 small ribonucleoprotein particles, the protein splicing (UA2F) factor and the arginine/serine rich (SR) proteins (Zahler et al., 1993; Chabot, 1996).

The results of the present study indicate that the primary site for regulation of LH receptivity through alternative splicing is the theca cells rather than the granulosa cells. A decrease in LH responsiveness, together with low circulating LH, deprives the growing follicles of LH-dependent factors such as androgens, and results in anoestrus.

In conclusion, the demonstration of expression of LH receptor during folliculogenesis in sheep indicates that follicular development is a relevant model for the study of the role of post-transcriptional events in reproduction. The characterization of such clear-cut, maturation-specific transcripts should form the basis for the design of diagnostic tests of terminal follicular growth that may be of value for the role of post-transcriptional events in reproduction. The characterization of translated products of the alternatively spliced luteinizing hormone receptor in the ovine ovary throughout the oestrous cycle Molecular and Cellular Endocrinology 147 113–124

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