Pituitary and Uterine Sex Steroid Receptors in Ewes

Seasonal and Postpartum Anoestrus, Oestrous Cycle and Experimentally Induced Subnormal Luteal Phases

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Abstract

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The general aim of this research was to gain knowledge of oestrogen and progesterone receptor (ER and PR) expression in the uterus and pituitary gland of the ewe in different reproductive stages (postpartum period, seasonal anoestrus and oestrous cycle), as well as in experimentally induced subnormal vs. normal luteal phases in anoestrous ewes.

Single, saturable and high-affinity binding sites for both oestrogen (E) and progesterone (P) were demonstrated in all of the tissue samples of the pituitary and the uterus. The values of the apparent dissociation constants (Kd) of ER and PR did not differ between the different postpartum days examined. Likewise the Kd values of ER and PR did not differ between anoestrous ewes, anoestrous treated ewes and cyclic ewes. The similar Kd values found during the different reproductive stages suggest that variations in the sensitivity of these target tissues to the ovarian hormones may not depend on changes in receptor affinity but rather on the binding capacity (number of receptors).

During the postpartum period of ewes lambing in the breeding season, both ER and PR concentrations in the uterus were significantly lower in early than in late postpartum. The correlation between PR and ER concentration was positive, while the correlation between uterine weight and the concentration of either steroid receptor was negative. During the late postpartum period the number of ewes with follicles larger than 4 mm (presumptive oestrogen-active follicles) increased. Therefore, the restoration of uterine ER and PR concentrations was temporally associated with the presence of E-active follicles in the ovary. Overall results suggest that E up-regulated the uterine steroid receptor concentrations and these molecular events may be involved in the uterine remodelling in the late postpartum period during the breeding season.

In seasonal anoestrous ewes, low pituitary ER and PR concentrations were found; in contrast with the high receptor concentrations found in the uteri of the same animals. However, the ERα mRNA concentrations in both the pituitary gland and the uterus were similar. While P treatment did not affect the pituitary receptor concentrations, it did decrease the uterine receptor concentrations, but it did not affect ERα mRNA concentrations in either the pituitary or the uterus. Treatment with gonadotrophin-releasing hormone (GnRH), with or without P in the anoestrous ewes, increased the pituitary ER and PR concentrations ten fold without affected the uterine receptor concentrations. GnRH treatment (with or without P) increased ERα mRNA concentrations in both the pituitary gland and the uterus. The decreases of uterine steroid receptor concentrations with P treatment, without affecting the ERα mRNA concentrations, suggest that P down-regulation occurs at posttranscriptional level. The results show that regulation of ER and PR concentration by P and GnRH is tissue specific in anoestrous ewes.

During the normal oestrous cycle in the breeding season, both pituitary and uterine ER and PR concentrations were higher on day 1 than on days 6 and 13 after oestrus. This higher steroid receptor concentration at the expected time of ovulation than in the luteal phase of the oestrous cycle is consistent with the known up- and down-regulation exerted by E and P respectively on receptor expression. The high pituitary steroid receptor expression found in cyclic and GnRH treated ewes as compared with anoestrous ewes suggest that this increase of sensitivity to the steroid hormones is needed for the pituitary gland to control the cyclic function.

Experimental subnormal or normal luteal phases were induced by GnRH or P + GnRH-treatments in anoestrous ewes. In all treated ewes, a synchronised surge of luteinizing hormone and follicle-stimulating hormone was found. The control animals treated with P + GnRH developed normal luteal phases and the GnRH-treated ewes developed subnormal luteal phases.

The pattern of pituitary steroid receptor concentrations in the P + GnRH-treated ewes resembled the pattern found during the normal oestrous cycle, with ER and PR concentrations decreasing from the expected time of ovulation (Day 1) to the early luteal phase (day 5 or 6). In contrast, in ewes treated with GnRH alone, pituitary ER and PR concentrations increased in the early luteal phase suggesting that this impaired expression of steroid receptors may be involved, in the development of subnormal luteal phases.

In the uterus, whereas in the GnRH-treated ewes the receptor concentrations increased from days 1 to 5, in the P + GnRH-treated ewes as well as in cyclic ewes the receptor concentrations decreased. On day 5, the GnRH-treated ewes had lower progesterone concentrations, and higher uterine ERα mRNA, ER and PR concentrations than the P + GnRH-treated ewes did. The results suggest that the induction of steroid receptor expression in the uterus and the hormonal environment found in the GnRH-treated ewes at the expected time of premature luteolysis may be involved in the mechanisms causing subnormal luteal phases.

Key words: sex steroid receptors, postpartum, anoestrous ewes, subnormal luteal phase.

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“El viento habla con igual dulzura a los gigantescos robles que a las hierbas más insignificantes; y solo es grande quien transforma la voz del viento en melodía, más dulce aún gracias a su capacidad de amar”.

Gíbran Jalil Gíbran

A Ambrosio
A Gonzalo
Su amor y apoyo son mi melodía.

Contents

Appendix

Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–IV).

I. Tasende, C., Meikle, A., Rubianes, E. & Garófalo, E.G. 1996. Restoration of estrogen and progesterone uterine receptors during the ovine postpartum period. *Theriogenology 45*, 1545–1551.

II. Tasende, C., Meikle, A., Rodríguez-Piñón, M., Forsberg, M. & Garófalo, E.G. 2002. Estrogen and progesterone receptor content in the pituitary gland and uterus of progesterone-primed and gonadotropin releasing hormone-treated anestrous ewes. *Theriogenology 57*, 1719–1731.

III. Tasende, C., Forsberg, M., Rodríguez-Piñón, M., Acuña, S. & Garófalo, E.G. 2005. Experimentally induced subnormal or normal luteal phases in sheep: reproductive hormones profiles and uterine sex steroid receptor expression. *Reproduction fertility and development 17, 565–571.*

IV. Tasende, C., Rodríguez-Piñón, M., Acuña, S., Garófalo, E.G. & Forsberg, M. 2005. Corpus luteum life span and pituitary oestrogen and progesterone receptors in cyclic and GnRH-treated anoestrous ewes*. In Press*.

Abbreviations

αERKO: oestrogen receptor-alpha knockout
βERKO: oestrogen receptor-beta knockout
αβERKO: oestrogen receptor-alpha and beta knockout
CL: corpus luteum
DNA: deoxyribonucleic acid
E: oestrogens
E2: oestradiol
ER: oestrogen receptor
ERα: oestrogen receptor subtype α
ERβ: oestrogen receptor subtype β
FSH: follicle-stimulating hormone
GnRH: gonadotrophin-releasing hormone
LH: luteinizing hormone
mRNA: messenger ribonucleic acid
OxR: oxytocin receptor
P: progesterone
PGF2α: prostaglandin F2α
PR: progesterone receptor
PR-A: progesterone receptor isoform A
PRAKO: progesterone receptor A knockout
PR-B: progesterone receptor isoform B
PRBKO: progesterone receptor B knockout
PRKO: progesterone receptor A and B knockout
RNA: ribonucleic acid

General introduction

Ovarian Steroid Hormones

Steroid hormones play a major role in the control of reproduction in mammals. They are directly involved in the events of the oestrous cycle leading eventually to ovulation and the formation of the corpus luteum (CL). In ewes, steroid hormones are also involved in the events responsible for the lack of ovulation during the postpartum and anoestrous seasons. These different physiological situations reflect the co-ordinated hormonal communication that exists among different tissues of the body (for a review, see Goodman, 1994).

Ovarian steroid synthesis

The ovarian follicles synthesize steroids from cholesterol. Most of these 1–2.5 mm diameter follicles (gonadotrophin-responsive follicles) contain androgens in the follicular fluid, androgens, which are produced by the theca cells. The aromatase activity, which converts androgens to estrogens in the granulosa cells, is induced in this class of follicles (for a review, see Scaramuzzi *et al.*, 1993). For a follicle to grow larger than 2.5 mm in diameter (gonadotrophin-dependent follicles), there is an absolute requirement for follicle-stimulating hormone (FSH), which induces aromatase activity. The follicles also require luteinizing hormone (LH) secretion for the production of androgen substrate for aromatization to oestradiol (for a review, see Driancourt & Thuel, 1998). As the gonadotrophin-dependent follicles continue to grow and become preovulatory follicles, there is a higher androgen output by the theca cells together with a higher aromatase activity in the granulosa cells. This activity, which is differentiated earlier, during the follicular phase, results in a high oestradiol output (for reviews, see Scaramuzzi *et al.*, 1993; Driancourt & Thuel, 1998). Formation of CL is initiated by morphologic and biochemical changes in the theca interna and granulosa cells of the preovulatory follicle: these changes are called “luteinization” (for a review, see Niswender & Nett, 1994). It is generally accepted that luteinization has a primary stimulus, the preovulatory LH surge. In sheep, theca cells persist as small luteal cells, whereas granulosa cells become large luteal cells after ovulation; both these types of cell secrete progesterone (P) (for reviews, see Niswender & Nett, 1994; Murphy, 2000). As the CL develops, P secretion increases: in the ewe, maximum concentrations are reached on day 8 after ovulation; concentrations remain constant up to day 14, and then rapidly fall due to the luteolytic effect of uterine Prostaglandin F2α (PGF2α) (for a review see Goodman, 1994).

From ovarian secretion to the target tissues

Both oestrogen (E) and P act on structures remote from the ovary. Since these molecules are small and fat-soluble, they circulate in the plasma bound loosely to serum albumin or to specific steroid-binding globulins with high affinity (for a review, see Clark *et al.*, 1992). The hormone bound to steroid-binding globulin is

in a dynamic equilibrium with a small quantity of free hormone in the plasma. Because oestrogen and progesterone are fat-soluble molecules, they are able to enter the cells by means of passive diffusion. When the free hormone enters the cell, a new small quantity of hormone is released again from the steroid-binding globulin (for review, see Clark *et al.*, 1992; for review see, Edqvist & Forsberg, 1997).

Mechanism of oestrogen and progesterone action

Even when the ovarian steroid hormones can reach all cells of the body, they are concentrated in the "target tissues" that have specific proteins named "receptors". Oestrogen and progesterone receptors (ER and PR, respectively), members of the nuclear receptor super family, function as ligand-activated transcription factors, regulating the synthesis of specific deoxyribonucleic acid (DNA), ribonucleic acids (RNAs) and proteins (for reviews, see Ing *et al.*, 1993; Clark & Mani, 1994). The ER and PR nuclear receptors are similar in their basic molecular structure to the other members of the nuclear receptor family, in that they are composed of independent but interacting functional domains. The N-terminal A/B domain enables the receptor to interact with members of the transcriptional apparatus. The C domain tightly binds the receptor to the DNA hormone response elements. The D domain, a binding domain, binds heat shock proteins and probably harbours the sequence representing the nuclear localization signal. The E/F multifunctional domain recognizes the ligand and is involved in receptor dimerization and interaction with transcription factors. The gene modulatory effect of the receptor, following the binding of a ligand, depends on the conformational changes of the receptor induced by the ligand and on subsequent events, including the release of heat shock proteins, receptor dimerization, receptor-DNA interaction, recruitment of the transcriptional machinery and interaction with other transcription factors to activate or repress target genes (Tsai & O'Malley 1994; for reviews, see Couse & Korach, 1999; Nilsson & Gustafsson, 2002).

In addition to the genomic action via nuclear receptors, E and P can exhibit non-genomic effects through specific receptors localized in the surface membrane in reproductive and non-reproductive tissues (for a review see, Revelli *et al.,* 1998). The E and P non-genomic actions are rapid and insensitive to transcription inhibitors (Bramley, 2003), while the genomic actions have a latency of several minutes, hours or days. The nature of the membrane steroid receptors and their physiological functions and interactions with the nuclear receptors are still subject to debate.

The diversity and cellular selectivity of effects displayed by E or P cannot be explained by a single mechanism of action. Recent advances in the discovery of new types of sex steroid receptors and co-regulators, which act as activators or repressors (Kuiper *et al.,* 1996; Conneely, 2001) contribute to a better understanding of the diverse mechanisms of steroid hormone action. Other recent contributions to the knowledge of steroid receptor functions include the generation of animals lacking ERs or PRs by disrupting their respective genes or products (Couse & Korach, 1999; Conneely *et al*., 2001; Hewitt & Korach, 2003).

Oestrogen receptors

In addition to the "classic" nuclear ER described and now named ER-alpha (ERα), a second subtype was discovered, named ER-beta (ERβ). These ERs are products of two different genes. There are differences between the distribution of ERα and of ERβ in the target tissues, and most of the available data has been generated in rodents. ERα is predominant in the reproductive tract, while ERβ is more abundant in the ovary (Kuiper *et al*., 1997; Couse & Korach, 1999; Wang *et al.*, 2000). In sheep, ERα and ERβ have been identified in hypothalamic cells (Scott *et al.,* 2000). ERα mRNA has been identified in the pituitary gland of prepubertal ewes (Meikle, 2001). Low pituitary ERβ expression was found in sheep (for a review, see Clarke, 2002). Both ERβ (Jansen *et al.*, 2001) and ERα (for a review, see Schams & Berisha, 2002) were identified in the ovaries of sheep. Uterine ERα was detected in ewes (Ing & Ott, 1999), and it is believed that it is the receptor protein that mediates the classical oestrogen action on the reproductive tract, as was suggested for rodents (Couse & Korach, 1999; Wang *et al*., 1999; Wang *et al*., 2000). Endometrial ERβ mRNA in sheep (Whitley *et al.,* 2000) has been described, and immunoreactive ERβ was found in lamb uteri (Morrison *et al.,* 2003).

Progesterone receptors

Progesterone receptors are expressed as two distinct isoforms, PR-A and PR-B that arise from a single gene by distinct promoters and by two alternative translation initiation signals. Both the isoforms are capable of dimerizing, interacting with the same DNA responsive elements and binding P with similar affinity (for a review, see Conneely *et al.,* 2000). Overall, it has been suggested that in the uterus, PR-A is responsible for the antioestrogenic action of P and PR-B for its proliferative effect, while in the mammary gland both PR-A and PR-B act as proliferative mediators of P action. PR-B may function as an activator whereas PR-A acts as a repressor of P-responsive genes and of the transcriptional activity of ERα (Conneely, 2001; Conneely *et al*., 2002). PR-A is necessary to elicit the P-dependent reproductive responses necessary for female fertility, while PR-B is required to elicit normal proliferative responses of the mammary gland to P (Mulac-Jericevic, *et al*., 2003). We were unable to find any reports of PR-A and PR-B isoforms in ovine, although, both isoforms have been described in the bovine oviduct (Ulbrich *et al.,* 2003).

Regulation of oestrogen and progesterone receptor expression

The presence of specific receptors is the primary determinant of tissue responsiveness to ovarian steroid hormones (Clark *et al*., 1992). The most powerful regulators of ER and PR concentrations in reproductive tissues are the ligands themselves. Ligand-receptor complexes are transcription factors that can activate or repress target genes, including the ER and PR genes. In most species, including sheep, it is accepted that E induces ER and PR transcription and synthesis, while P down-regulates both receptors (Ing *et al.,* 1993; Clark & Mani,

1994). The up- and down-regulation exerted by E and P on the ER and PR, respectively, was demonstrated in the myometrium of adult ovariectomized ewes treated with oestradiol (E2) or P (Rexroad, 1981b). However, E2 treatment of entire prepubertal lambs increased uterine ERα and PR mRNA concentrations, but decreased their binding activities (Meikle *et al.*, 1997, 2000). An E2 down-regulation of ER expression was also found in rat uteri, and this regulation is dose dependent, as demonstrated by the stimulating or inhibitory effects of low or high doses of E2, respectively (Medlock *et al*., 1994). An up-regulatory effect of E2 on the pituitary PR mRNA expression of both the A and B isoforms was found in ovariectomized E2-treated rats, but P treatment did not affect the concentrations of PR mRNA (Szabo *et al*., 2000).

In ovariectomized ewes, E2 treatment increases endometrial ER mRNA and PR mRNA concentrations, and nuclear runoff analysis showed that whereas E2 enhances the transcription rates of PR, transcription rates of the ER gene remained unchanged (Ing *et al*., 1996). These results suggest that E2 up-regulates ER gene expression by a posttranscriptional mechanism (Ing *et al*., 1996). E2 enhanced ER mRNA stability (half life increased from 9 to ≥ 24 h); thus E2 up-regulates the steady-state of endometrial ER mRNA by means of a posttranscriptional mechanism (Ing & Ott, 1999). The down-regulation of steroid receptors may be the consequence of inhibiting the synthesis or stimulation of receptor inactivation and/or degradation. A decrease in ERα protein concentration was demonstrated by enzyme immunoassay in E2-treated prepubertal ewes, suggesting that the initial decrease in binding capacity was due to a loss of the protein itself, rather than to receptor inactivation (Meikle *et al.,* 2000). The E2-dependent steroid receptor down-regulation may be the result of receptor processing (Zhou *et al.*, 1993) and/or degradation by specific proteases (Alarid *et al.*, 1999; Preisler-Mashek *et al*., 2002). In addition, P treatment of prepubertal lambs down-regulated ER and PR (Meikle *et al.,* 1997). The ovarian steroid receptors present a complex control mechanism in which it is necessary to consider the different receptor types and their selective expression in target tissues, as well as the hormonal status in the different physiological reproductive stages.

Physiological reproductive stages studied in this thesis

The ovine oestrous cycle

Sheep are seasonal breeders and their reproductive pattern is influenced by photoperiod. During the non-breeding season (anoestrus) ovulations usually cease, but in the breeding season regular oestrous cycles occur with 16–18 days between ovulations (for a review, see Goodman, 1994). The oestrous cycle in the sheep is co-ordinated by hormonal interaction between the brain (gonadotrophin-releasing hormone, GnRH), the pituitary gland (LH and FSH), the ovary (follicles: oestrogen and inhibin – E and I, respectively; CL: progesterone and oxytocin – P and Ox, respectively) and the uterus (PGF2α) (for a review, see Goodman, 1994). Ovarian steroid hormones play a major role in the control of this cycle acting through their corresponding receptors in the above-mentioned tissues.

ERα and ERβ as well as PR have been localized in the hypothalamic neurons of sheep, suggesting that steroids are involved in the regulation of GnRH secretion (Scott *et al*., 2000). We are unaware of any reports of the presence of ER in GnRH neurons in sheep, but it was demonstrated in rats that GnRH neurons contain ERβ (Petersen *et al*., 2003; Abraham *et al*., 2003). Thus, the presence of ER in the GnRH neurons of sheep cannot be ruled out.

In sheep, an increase in pulsatile GnRH secretion drives the preovulatory LH surge in a dose-dependent fashion, and the amplitude of GnRH surge may exceed that needed to generate the LH surge (Bowen *et al*., 1998). Oestrogen and P also modulate the expression and secretion of gonadotrophins by the pituitary gland; E strongly inhibits FSH synthesis by blocking transcription of both subunit genes (alpha and beta). Concentrations of LHβ mRNA were unaffected by E alone, but are decreased dramatically by a combination of E plus P *in vivo*, suggesting that P has a preponderant role in the synthesis of LH (Miller, 1993). Progesterone may directly inhibit pituitary LH secretion in an E2-dependent manner (Girmus & Wise, 1992). Pituitary sensitivity to oestrogen – in terms of number of gonadotroph cells ERα positive determined by immunostaining – was reported to experience cyclic changes during the ovine oestrous cycle, being higher during the follicular phase (Tobin *et al*., 2001). As mentioned previously, it is accepted that P acts directly on the pituitary gland through a receptor-mediated mechanism that regulates gonadotrophin secretion; however, the pattern of pituitary PR during the oestrous cycle of the ewe had not been described when this thesis was written.

Uterine cyclic changes in ER and PR concentrations, as determined by ligand-binding assays (Miller *et al*., 1977; Rexroad, 1981a), have been demonstrated during the ovine oestrous cycle. The uterine ER and PR concentrations are higher at oestrous than in the luteal phase. ERα and PR transcript expression during the ovine oestrous cycle agrees with the receptor dynamics (Ott *et al*., 1993). The pattern of steroid receptor concentrations in the uterus correlate with circulating ovarian steroid hormone concentrations: the high E2 concentrations around the time of oestrus up-regulate the receptor concentrations, while during the luteal phase, P down-regulates their expression. In addition, it was demonstrated by immunohistochemistry that the regulation of ER and PR levels is cell-type specific (Cherny *et al*., 1991; Spencer & Bazer, 1995; Sosa *et al*., 2004). The uterine ER and PR contents were high shortly after oestrus in the different compartments, but then declined to negligible levels by the mid luteal phase except in deep caruncular stroma (Cherny *et al*., 1991). Thus, in general ER and PR distribution in the different uterine compartments varies cyclically, correlating with steroid hormone levels, although individual cell types can display differential sensitivities to oestrogen and progesterone (Cherny *et al*., 1991). In ewes and cows, the establishment of the positive feedback mechanism between endometrial PGF2α and ovarian Ox terminates the life of the corpus luteum, allowing a new cycle to begin (Flint *et al*., 1992; Wathes & Denning-Kendall, 1992). The release of luteolytic PGF2α from the endometrium is regulated by E and P (for reviews, see McCracken *et al*., 1999; Okuda *et al*., 2002; Goff, 2004). E2 and P modulate PGF2α secretion by regulating the concentration of OxR (McCracken *et al*., 1999): P down-regulates OxR, delaying the time of luteolysis; while E2 up-

regulates OxR, advancing luteolysis (Wathes & Lamming, 1995; McCracken *et al*., 1999).

Postpartum period

Postpartum may be considered as the period from parturition to first oestrus. The onset of ovarian cyclicity after parturition is affected by season, breed, nutrition and lactation (for a review, see Novoa, 1984). Studying the postpartum ewe during the non-breeding season limits the possibility of gaining an understanding of the mechanisms involved in this period, due to overlapping with seasonal anoestrus. Because of that, we will examine the data obtained when ewes lamb during the breeding season.

The most important processes that take place in the postpartum period are regeneration of the endometrium, uterine involution and resumption of ovarian cyclicity. In the early postpartum period the release of pituitary LH is greatly reduced (Wright *et al.*, 1983; Clarke *et al.*, 1984); in spite of this, the hypothalamic GnRH and pituitary GnRH receptor concentrations seem to be sufficient to maintain LH secretion (Crowder *et al.*, 1982). Wise *et al.* (1986) reported that pituitary and hypothalamic oestrogen receptor concentrations were low during late gestation and remained low in the early postpartum, suggesting low hypothalamic-pituitary sensitivity to oestradiol. This may explain the low circulating gonadotrophin concentrations found (Schirar *et al*., 1990) and the presence of small follicles at the ovarian surface at this time (Tsonis *et al.,* 1984; Driancourt, 1991; Rubianes & Ungerfeld, 1993). During the late postpartum period an increase in GnRH pulse frequency was observed (Wise, 1990). The pituitary and hypothalamic oestrogen receptor concentrations also increased at this time (Wise *et al.,* 1986), suggesting that the sensitivity to oestradiol is recovered; this could explain the increased gonadotrophin secretion observed in this period (Schirar *et al*., 1990). This is consistent with the presence of large active oestrogen-secreting follicles on the ovarian surface and with the occurrence of ovulation (VanWyck *et al.,* 1972; Rubianes & Ungerfeld, 1993). Usually, macroscopic uterine involution and cyclic ovarian activity in sheep are accomplished at about three to four weeks postpartum (Mallampati *et al*., 1971; Rubianes & Ungerfeld, 1993). Frequently, the re-establishment of ovarian cyclicity post partum is associated with inadequate or subnormal luteal phases, due to the development of CL of short lifespan or CL of normal lifespan but decreased P secretion (Wright *et al.*, 1983; for a review, see Goodman, 1994).

Seasonal anoestrus

During the anoestrous season the size range and numbers of ovarian antral follicles are similar to those seen during the breeding season, (Ravindra & Rawlings, 1997), but ovulation does not occur (for a review, see Goodman 1994). The change in the reproductive status during the anoestrous season is controlled by modifications in the activity of the gonadotrophic axis through variation in pulsatile LH secretion (for a review, see Gallegos-Sanchez *et al.,* 1998).

The lack of ovulation during anoestrus is caused by a decreased frequency of pulsatile LH secretion, which is the result of the increased sensitivity of the hypothalamic-pituitary axis to the negative feedback action of E2 (Karsch *et al*., 1980). Differences in the sensitivity of the hypothalamic-pituitary axis to the negative feedback action of E2 could be due to variations in the concentrations of ER. Indeed, Wise *et al*. (1975), found that the concentration of ER in the pituitary glands of ovariectomized ewes is greater during anoestrous than in the breeding season, which contradicts the findings of Glass *et al*. (1984) using the same experimental model. Clarke *et al*. (1981) could not demonstrate any seasonal variation in the pituitary ER concentrations in sheep, since anoestrous ewes and cyclic ewes in the luteal phase had similar ER concentrations. During the anoestrous season, the follicular growth (Souza *et al.*, 1996; Ravindra & Rawlings, 1997) and ovarian steroid secretion in sheep occurs in wave-like patterns (Souza *et al.*, 1996). The LH pulse frequency, mean and basal serum concentrations increased in late anoestrus, but no major trends in the serum concentrations of FSH and E2 were seen during this period (Ravindra & Rawlings, 1997). At the end of the anoestrous season, an LH surge resulted in a short-lived secretion of P (inadequate or subnormal luteal phases) that was followed by the first observed ovulation and the first ovulatory cycle of the breeding season (Ravindra & Rawlings, 1997).

Subnormal luteal phase

The CL is a transient endocrine organ and its primary function is to secrete P, a hormone that is an important regulator of oestrous cycle length and essential for the maintenance of pregnancy (for a review see Niswender & Nett, 1994). The CL is controlled by hormones which play a crucial role in providing the signals for luteotrophic support during the oestrous cycle and pregnancy and for the induction of luteolysis at the end of the oestrous cycle (for a review see Milvae *et al*., 1996).

Understanding the factors that regulate the lifespan and function of the CL could have a major impact on limiting reproduction, since 25–55% of all mammalian embryos are lost during early gestation and much of this loss appears to be caused by subnormal luteal phases (for a review, see Niswender & Nett, 1994). Subnormal luteal phases naturally occur in ewes when reproductive activity is being re-established after postpartum, or after seasonal anoestrus or at the onset of puberty, and are characterized by a short lifespan CL and/or subnormal concentrations of circulating P (Keisler *et al*., 1983; Hunter, 1991; Ravindra & Rawlings, 1997).

Similarly, a subnormal luteal phase is seen following induction of ovulation after treatment with multiple small doses of GnRH in anoestrous ewes (Hunter, 1991; Garverick *et al*., 1992). However, combined treatment with P and GnRH ensures that normal luteal phases occur (McLeod *et al*., 1982; Southee *et al*., 1988). This suggests that previous exposure to P is necessary for normal luteal phases (for reviews, see Hunter, 1991; Goodman, 1994)

A preovulatory LH peak occurs spontaneously in seasonally anoestrous ewes treated with small doses of GnRH, but the interval from the start of the GnRH

injection to the onset of the preovulatory LH surge is longer in P-pre-treated ewes than in animals not pre-treated with P (McLeod *et al*., 1982; Southee *et al*., 1988). However, it has been suggested that it is not this extended period of LH exposure of the follicles that is responsible for the functional competence of the resultant CL, since when an LH preovulatory peak is induced earlier by a bolus injection of GnRH in P-treated anoestrous ewes, all ewes develop a normal luteal phase (McLeod & Haresign, 1984). It was reported that the peak concentration of the GnRH-induced LH surge was higher and the interval from GnRH to peak LH discharge was shorter in ewes with a subnormal CL than in ewes with a normal CL (Bartlewski *et al.*, 2001). Similarly, treatment with GnRH alone induced a higher LH peak than did a combined treatment of progestagen + GnRH; the GnRH treatment started immediately after progestagen withdrawal (Bartlewski *et al.*, 2004). However, when GnRH treatment started 1 day after progestagen withdrawal, no differences in the GnRH-induced LH peak were found (Bartlewski *et al.* 2004). Overall, the results suggest that gonadotrophin hormones are involved in determining the subnormal or normal luteal phase. Considering that the aforementioned studies used exogenous GnRH treatment, the pituitary gland may be involved in determining the type of subsequent luteal phases.

The mean number of follicles ≥3 mm in diameter at the surface of the ovary did not differ between P-pre-treated and untreated postpartum cows before GnRH treatment (Garcia-Winder *et al*., 1987). However, after ten hours of GnRH treatment, follicular diameters as well as E2 concentrations in the P-pre-treated cows increased while both follicular diameters and E2 concentrations remained unchanged in the controls (Garcia-Winder *et al*., 1987). In sheep and cattle, preovulatory secretion of E2 was lower in animals developing short rather than normal luteal phases (Garcia-Winder *et al*., 1986; Garverick *et al*., 1988; for a review, see Garverick *et al*., 1992). On the other hand, no differences in the characteristics of the follicular wave or in the number of large follicles among progestagen + GnRH-treated and GnRH-treated ewes were found when GnRH treatment started immediately after progestagen removal (Bartlewski *et al.*, 2004). However, when GnRH treatment started one day after progestagen withdrawal, the number of large follicles in GnRH-treated ewes was higher than in progestagen + GnRH-treated ewes (Bartlewski *et al.*, 2004). Differences in the ovarian response may be due to the stage of follicle development at time of GnRH treatment and/or differences in gonadotrophic stimuli to the follicle.

The lifespan of subnormal, induced CLs in the breeding season was maintained in hysterectomized ewes (Moor *et al*., 1966). Hysterectomy also prevented the regression of CLs anticipated to have short lifespans in prepubertal ewes (Keisler *et al*., 1983) as well as in anoestrous ewes and postpartum cows (for reviews, see Hunter, 1991; Garverick *et al*., 1992). Therefore, as in CLs of normal lifespan, the uterus influences the lifespans of subnormal CLs. The destruction of the normal CL at the end of the oestrous cycle in nonpregnant ewes is brought about by the pulsatile secretion of endometrial PGF2α (for a review see, Niswender & Nett, 1994). This increase in the PGF2α pulsatility must be co-ordinated with an increase in the number of uterine oxytocin receptors (OxR). The release of luteolytic PGF2α from the uterus is regulated by E and P, acting through their corresponding receptors (ER and PR, respectively) (McCracken *et al*., 1999; Goff,

2004). In the ewe with a subnormal luteal phase induced by GnRH treatment, an association between a major peak of oxytocin and a rise in PGF2α metabolite (PGFM) on days 3 or 5 after the end of GnRH treatment was found (Hunter *et al*., 1989). Moreover endometrial oxytocin binding sites were present in ewes that had not been pre-treated with P (Hunter *et al*., 1989). This suggests that the premature regression of subnormal CL occurs via the normal luteolytic mechanism, and that P pre-treatment can influence the production of oxytocin and its receptors (Hunter *et al*., 1989; for a review, see Hunter, 1991. When the experimental work of this thesis was initiated, no data was available regarding ER or PR uterine expression in ewes with induced subnormal vs. normal luteal phases.

The present study

Outline and aims of the study

A cell's responsiveness to ovarian steroid hormones (E and P) is related to the number and affinity of its receptors. Thus, factors that affect the number of steroid receptors may influence tissue sensitivity and functionality. The general aim of this investigation was to gain knowledge of oestrogen and progesterone receptor expression in the uterus and pituitary gland during different reproductive stages in the ewe: postpartum period, seasonal anoestrus and oestrous cycle as well as in experimentally induced subnormal vs. normal luteal phases in anoestrous ewes. The relationship between receptor expression in the uterus and pituitary gland and other endocrine and physiological events, such as the concentrations of circulating sexual hormones, was addressed in an attempt to clarify the role of oestrogen and progesterone receptors (ER and PR, respectively) in female reproductive physiology in sheep.

In Corriedale ewes lambing during the breeding season, cyclic ovarian activity and macroscopic uterine involution are accomplished at around three weeks postpartum (Rubianes & Ungerfeld, 1993). Although profiles of E and P – the main regulators of uterine function – change during the postpartum period, it is not known whether uterine sensitivity to these hormones – in terms of steroid receptor concentrations – is affected by the biological changes that take place during the postpartum period. At parturition, very low myometrial ER and PR concentrations are found, suggesting a loss of myometrial sensitivity to E and P (Klauke & Hoffman, 1992). Therefore, we tested the hypothesis that uterine oestrogen and progesterone receptor concentrations could be modified, in relation to the restoration of ovarian cyclicity and uterine involution during the postpartum period, in ewes lambing in the breeding season (**Paper I**).

Short luteal phases or luteal phases with lower P concentrations (e.g., subnormal luteal phases) naturally occur at the initiation of cyclic activity following postpartum or seasonal anoestrus, and at the onset of puberty. Similarly, subnormal luteal phases are found following induction of ovulation by administration of multiple small doses of GnRH to anoestrous ewes. However, combined treatment with P + GnRH ensures normal luteal phases (McLeod *et al.*, 1982; Southee *et al.*, 1988); thus, previous exposure to P is necessary for normal luteal phases (for a review, see Hunter, 1991). Causes of subnormal luteal phases may include inadequate gonadotrophin secretion, impaired follicular development and/or premature luteolysis (for a review, see Garverick *et al*., 1992), and some of these causes were addressed in this thesis (**Papers II, III** and **IV**).

The concentrations of circulating LH (**Papers II** and **III**) and FSH (**Paper III**) as well as follicular status at slaughter (**Papers II** and **III**) were investigated in GnRH-treated ewes (subnormal luteal phases) and P + GnRH-treated ewes (normal luteal phases). The preovulatory LH surge in anoestrous ewes treated with GnRH has been reported to occur later in P-primed ewes (McLeod *et al.*, 1982; McLeod & Haresign, 1984). The peak concentration of the GnRH-induced LH

surge was higher in ewes with a subnormal CL than in ewes with a normal CL (Bartlewski *et al.*, 2001). In spite of this, no differences were found in GnRH-induced LH surges between GnRH- and P + GnRH-treated ewes (**Paper II**). In the subsequent study (**Paper III**), LH surges were determined and GnRH-treated ewes were found to have higher LH surges than P + GnRH-treated ewes did. Since in the aforementioned studies exogenous GnRH was given to anoestrous ewes, this suggests that the pituitary gland is involved in determining the type of the subsequent luteal phases. Differences in GnRH-induced LH surges in GnRH- and P + GnRH-treated ewes could be due to alterations in pituitary sensitivity to E and P (e.g., receptor concentrations); in view of this, ER and PR concentrations were determined in the pituitary gland (**Papers II** and **IV**).

LH does not only play an important role in the ovulatory follicle of sheep around the time of ovulation; following ovulation, the formation and function of the CL is dependent on pituitary gonadotrophin support (Miller *et al*. 1993; Niswender *et al*. 2000): LH stimulates P synthesis and secretion by the CL (Niswender & Nett, 1994). Since steroid ovarian hormones may control the release of pituitary gonadotrophin, to maintain CL function, pituitary ER and PR concentrations in anoestrous ewes treated with GnRH, either with or without P priming, were studied during the early luteal phase (**Paper IV**). To get a reference point and since pituitary PRs in the ovine oestrous cycle have not previously been described, pituitary ER and PR concentrations in the ovine oestrous cycle were also determined at the time of ovulation and during the early luteal phase (**Paper IV**).

As mentioned above, the other cause of subnormal luteal phase is premature luteolysis (for a review, see Garverick *et al*., 1992). In ewes and cows, luteolysis of normal and subnormal CL is prevented by hysterectomy; therefore the uterus influences the length of the luteal phase (for a review, see Garverick *et al*., 1992). Premature regression of subnormal CLs may be caused by the premature release of uterine PGF2α (on days 3–5) (for a review, see Hunter, 1991). The release of luteolytic PGF2α from the uterus is regulated by E and P, acting through their corresponding receptors (ER and PR) (McCracken *et al*., 1999; Goff, 2004). It was shown that cows expected to have short luteal phases had lower uterine PR concentrations than did cows with normal luteal phases; this suggests that the premature luteolysis is due to a diminished P dominance in the uterus (Zollers *et al.*, 1993). PR expression depends at least in part on oestrogenic actions (Ing *et al*., 1993), and no data concerning uterine ER during subnormal luteal phases in cows were found. Moreover, no such data have been reported in sheep. We hypothesized that ewes treated only with GnRH (subnormal luteal phases) will have an altered expression of uterine sex steroid receptors when compared to those treated with P + GnRH (normal luteal phases) at the expected time of ovulation (**Paper II**). Determination of receptor concentrations in ewes before initiation of the GnRH treatment (–P or +P anoestrous ewes) was also included in this experiment. Since E2 and P are the main regulators of ER and PR expression, the circulating concentrations of these hormones were also determined (**Papers II–IV**).

At the expected time of ovulation (day 1 after GnRH bolus injection), GnRH-treated ewes had higher uterine PR concentrations than did P + GnRH-treated ewes (**Paper II**). In contrast, on day 5 following the first postpartum ovulation, cows expected to have short luteal phases had lower uterine PR concentrations than did cows with normal luteal phases (Zollers *et al*., 1993). Therefore, in **Paper III** the uterine ER and PR and ERα mRNA concentrations, and the circulating concentrations of steroid ovarian hormones, in ewes treated with GnRH or P + GnRH (subnormal or normal luteal phases, respectively) were studied at Day 5 after GnRH bolus injection (expected time of premature luteolysis).

Materials and Methods

Experimental designs

The studies were carried out in Uruguay (30° to 35° LS). All animals were of the Corriedale breed. In the first and second studies, the animals were located at the Faculty of Veterinary Medicine, University of Uruguay, in Montevideo (**Papers I** and **II**), while the third and fourth studies were carried out at the experimental field station of the Faculty of Veterinary Medicine, University of Uruguay, in Migues (**Papers III** and **IV**). The ewes were kept under natural day length and given water *ad libitum*; they were either offered a maintenance diet of concentrate (**Papers I** and **II**) or were grazed on native pastures (**Papers III** and **IV**). All animal experimentation was performed in compliance with regulations established by the Faculty of Veterinary Medicine, University of Uruguay.

Paper I

Ewes were bred after progestogen and eCG treatment (Rubianes & Ungerfeld, 1993), so that lambing would occur during the normal breeding season, thus minimizing confounding effects of photoperiod on postpartum return to oestrus. Uterine tissues of ten multiparous ewes were studied after ovariohysterectomies on day 1 (n = 2), 5 (n = 4), 17 (n = 2) or 30 (n = 2) post partum. Blood samples were collected from ewes by jugular venipuncture three times per week, from parturition until ovariohysterectomy. Corpora lutea and follicles on the ovarian surface at time of ovariohysterectomy were recorded, and progesterone profiles were determined to evaluate restoration of ovarian activity.

Paper II

The experiment was performed during the mid-anoestrous season. Anoestrous condition of ewes was confirmed using vasectomized rams fitted with colour-marking harnesses, which were kept with the ewes for 2 months before the start of the study. Fifteen adult ewes were randomly assigned to four groups and treated as follows: Group C (control), not treated (n = 4); Group P, treated with 0.33 g of P (Controlled Internal Drug Release (CIDR), EASI-BREED®, Hamilton, New Zealand) for 10 days (n = 4); Group GnRH, treated every 2 h for 18 h with 6.7 ng i.v. GnRH (busereline acetate – Receptal®; Hoechst, Buenos Aires, Argentina) followed by a bolus administration of GnRH (4 μg Receptal) at 20 h (n = 4); Group P + GnRH, given the combined treatment of the P and GnRH groups (n = 3). GnRH treatment started immediately after CIDR removal, and the time of the GnRH bolus administration was set as 0 h. The GnRH treatment administered was similar to that used by McLeod *et al*. (1982) for the induction of ovulation in anoestrous ewes, and the dose of busereline acetate was calculated taking into account the fact that busereline acetate is approximately 40 times more potent than native GnRH is (Nawito *et al.*, 1977; Chenault *et al.*, 1990). The bolus treatment of GnRH was used to synchronize the onset of the preovulatory LH surge (Hunter *et al*., 1988). Blood samples were collected three times during P treatment and

every 2 h immediately before each GnRH treatment, from the first treatment until the bolus was given. Thereafter, samples were collected every 1 h for 6 h and then every 2 h until 24 h after the bolus treatment. Ewes were slaughtered as follows: at the beginning of the experiment (Group C), immediately after CIDR removal (Group P), or on day 1 after the bolus treatment (Groups GnRH and P + GnRH).

Paper III

Thirty-two adult anoestrous ewes (anoestrous condition was confirmed as in **Paper II**) were randomly assigned to two groups, namely, the GnRH group (n = 16) and the P + GnRH group (n = 16). The GnRH and P + GnRH groups were given the same treatment as the GnRH and P + GnRH groups, respectively, in the study reported in **Paper II**. Both treatments were followed by a bolus injection of GnRH at 18 h (0 h). The ewes treated with GnRH alone were expected to develop subnormal luteal phases (Southee *et al.,* 1988), while the P-pre-treated ewes were expected to develop normal luteal phases (Hunter, 1991). Five ewes from each group were used as controls for each treated group, to allow determination of the length of the luteal phase, judged by the P serum concentration over the course of 18 days (P + GnRHc, n = 5, and GnRHc, n = 5). The luteal phase was defined as normal when the concentrations of circulating P were >4 nmol/L for 12 days. The remaining ewes were slaughtered on Day 1 (n = 6 for each treatment) or Day 5 (n = 5 for each treatment) after the GnRH bolus injection. Blood samples for hormone determinations were collected every 2 h immediately before each GnRH injection, from the first injection until the bolus was given. Thereafter, samples were collected every 1 h for 6 h, then every 2 h for 6 h, and finally either every 4 h for 12 h in the case of ewes slaughtered on Day 1 after bolus treatment, or every 4 h for 24 h and then every 12 h for 120 h after the bolus treatment in the case of ewes slaughtered on Day 5 after bolus treatment. In the P + GnRHc and GnRHc groups, samples were collected three times for the 10 days of P pre-treatment (6, 3 and 1 days prior to GnRH bolus treatment), then daily for 8 days and thereafter on days 9, 12, 15, and 18 after bolus treatment.

Paper IV

Two experiments were conducted: experiment 1 was carried out during the breeding season (end of February to the beginning of March) and experiment 2, during the mid-anoestrous season (September). In experiment 1, nineteen ewes were used. Oestrus was synchronized using two doses of a $PGF_2\alpha$ analogue administered intramuscularly (i.m.) (150 μg, Glandinex®, Laboratorio Universal, Montevideo, Uruguay), 6 days apart. From day 10 of the first oestrous cycle, ewes remained with two vasectomized rams with marking crayons and were checked twice a day for service marks indicative of oestrus (day of oestrus = day 0). The ewes were slaughtered on days 1 (n = 7), 6 (n = 6) or 13 (n = 6) after oestrus detection. Blood samples for P and E2 determinations were collected at the time of slaughter. In experiment 2, twenty-two anoestrous ewes were used. The animals in experiment 2 (sacrificed on days 1 or 5) were the same ones used in the study reported in **Paper III** (GnRH-treated group, n = 11; P + GnRH-treated group, n = 11).

Comments on methods

Tissue and blood sampling procedures

The day before initiation of GnRH treatment and blood sampling, the animals were fitted under local anaesthesia with an indwelling jugular catheter for collection of the samples (**Papers II** and **III**). In all P-treated ewes, blood samples were taken by jugular venipuncture before and during the P treatment (**Papers II** and **III**), three times per week (**Paper I**) and at the time of slaughter (**Paper IV)**. Blood samples were centrifuged within the first hour of collection and serum was stored at –20°C until hormone assays were performed.

At time of ovariohysterectomy (**Paper I**) or at slaughter (**Papers II–IV**), the uteri, (**Papers I–IV**), pituitary glands (**Papers II** and **IV**) and ovaries (**Papers I, II** and **III**) were dissected at a temperature of 0−4°C and then weighed. To obtain uniform samples of whole uteri (including myometrium, endometrium and caruncles), we selected the upper portions of the uterus, defined as the portion of the uterine horn next to the oviduct. The tissues were frozen in liquid nitrogen and stored at –80°C until binding assays of ER and PR (**Papers I–IV**) and solution hybridization ERα mRNA were performed (**Paper III** and Tasende et al., unpublished data).

Examination of the ovaries at time of slaughter

The numbers of follicles present on the ovarian surfaces were recorded and the follicles were classified according to diameter as "small" (≤2 mm), "medium" (>2−4 mm) and "large" (>4 mm) (Scaramuzzi *et al*,. 1993; Driancourt & Thuel, 1998). The numbers of ruptured follicles and CLs were also recorded (**Papers I**, **II** and **III**). The follicles 1 to 2 mm in diameter are gonadotrophin responsive and the follicles >2 mm in diameter are gonadotrophin-dependent follicles (Scaramuzzi *et al*., 1993). The follicles >4 mm in diameter have increased aromatase activity and thus are oestrogen-active follicles (Tsonis *et al*., 1984; Driancourt & Avdi, 1993).

Oestrogen and progesterone receptor binding assays

The uterine (**Papers I–IV**) and pituitary (**Papers II** and **IV**) ER and PR determinations were performed by means of ligand-binding assays in the cytosolic fractions. In the binding assays, both ERα and ERβ types are measured but not discriminated. The ERs and PRs are situated predominantly in the nuclei of the cells (Perrot-Applanat *et al.*, 1992). The term "cytosolic receptors" represents the receptor concentration measured in the supernatant fraction after cellular disruption during homogenization of the uterine tissues and after high-speed centrifugation (Martin & Sheridan, 1982). Previously, we determined ER and PR content in both the cytosolic and nuclear fractions, but less than 10% of the total receptor content corresponded to the nuclear fraction (Meikle *et al*., 2000). The labelled ligands used were ^{3}H-E2 for ER (86 Ci/mmol: 0.15–15 nM or ^{3}H-ORG-2058 (40 Ci/mmol: 0.25–30 nM) for PR while the nonlabelled ligands used were diethylstilbestrol and ORG-2058, respectively (**Papers I–IV**). Linear regression of inverse Scatchard model analysis of the data was performed (Braunsberg, 1984).

This provided the apparent dissociation constant (Kd), and the concentration of receptor sites at the intercept, Bmax, expressed in fmol/mg protein and fmol/mg tissue. Proteins concentrations were measured using the method of Lowry *et al.* (1951).

Hormone determination

All hormone determinations were done by radioimmunoassay (RIA) (**Papers I–IV**). Progesterone was assayed using a direct solid-phase RIA method previously described for use with ovine serum (Garófalo & Tasende, 1996) (**Papers I–IV**). Oestradiol-17β determinations were performed using a liquid-phase RIA method previously described for use with ovine serum (Meikle *et al.*, 1997) (**Papers II–IV**), as were LH (Forsberg *et al.*, 1993) (**Papers II** and **III**) and FSH (Meikle *et al.*, 1998) (**Paper III**) determinations.

Solution hybridization assay of ERα mRNA

Determination of ERα mRNA was performed in uteri (**Paper III**, Tasende *et al*., unpublished data) and pituitary glands (Tasende *et al*., unpublished data) using the solution hybridization method (Meikle *et al*., 2000). The hybridization probes used for ERα mRNA determinations were derived from plasmids containing 360 bp cDNAs from the ovine ERα (oERα), generously supplied by Dr. N. Ing (Texas A & M University, TX, USA) (Ing *et al.,* 1996). Probes were synthesized *in vitro* and radiolabelled with ^{35}S-UTP as described by Melton *et al.* (1984). Restriction of the vector (pGEM4Z) containing a fragment of the oERα cDNA with EcoRI allows the synthesis of an antisense RNA probe using T7 RNA polymerase. Total nucleic acids (TNA) were prepared by digesting homogenized tissues with proteinase K in buffer containing SDS, followed by subsequent extraction with phenol–chloroform. The concentrations of DNA in the TNA samples were measured spectrophotometrically at a wavelength of 260 nm. All sets of tissue samples were run in one assay. The ERα mRNA concentrations were calculated as amol/μg DNA and expressed as percentages of the corresponding controls.

Statistical analyses

Student's t tests were performed to compare uterine receptor concentrations and Kd values between early and late postpartum groups (Statgraphics, Statistical Graphics Corp., USA) (**Paper I**). The weights of the uteri (**Papers II–IV**) and pituitary glands (**Papers II and IV**), the numbers of small, medium and large follicles (**Papers II and III**), the Kd values, uterine receptor concentrations (**Papers II–IV**), ERα mRNA concentrations (**Paper III**) and E2, P (**Papers II III and IV**), LH (**Papers II and III**) and FSH (**Paper III**) serum concentrations were analyzed by analysis of variance (ANOVA) using the MIXED procedure of Statistical Analysis Systems (SAS Institute Inc., Cary, NC, USA). The statistical model included the effects of time of sampling (for hormones) or day of sampling (for receptors and follicular data) and treatment (for P + GnRH and GnRH treatments) (**Papers II–IV**), and the interactions between them. The statistical model for data for ewes during the oestrous cycle included only the effect of day

of sampling (**Paper IV**). The correlation procedure available in SAS was performed to study the relationships between ER and PR concentrations (**Papers II–IV**), between uterine weight and receptors concentrations (**Paper I**). The number of ewes with large follicles (**Paper I**), ruptured follicles (**Papers II and III**) and CLs (**Paper III**) at slaughter was compared using the chi-square test. The level of significance was $P<0.05$. The LH surges were characterized in terms of the interval between the GnRH bolus administrations and the beginning of the LH release, duration of LH discharge, and maximum LH concentration (**Papers II** and **III**).

Results

A single, saturable and high-affinity binding site for E or P was demonstrated in all samples of pituitary (**Papers II** and **IV**) and uterine tissues (**Papers I–IV**). The Kd values of ER and PR did not differ on different days postpartum (**Paper I**). Likewise, the Kd values of ER and PR did not differ between anoestrous ewes (**Paper II**), anoestrous treated ewes (**Papers II–IV**) and cyclic ewes (**Paper IV**).

Both ER and PR concentrations in early post partum (days 1 to 5) were significantly lower than in late postpartum (days 17 to 30). There was a positive correlation between PR and ER concentration and negative correlations between uterine weight and both steroid receptor concentrations. While only one of six ovarihysterectomized ewes had a follicle larger than 4 mm (presumptive oestrogen-active follicle) on days 1 to 5 post partum, four of four ovarihysterectomized ewes presented follicles of this size on days 17 to 30. The ovary of one of the ovarihysterectomized ewes showed a regressed CL (7 mm in diameter) on day 30 post partum. The duration of this luteal phase (8 days) was shorter than normal. While this ovarihysterectomized ewe showed an increase in serum progesterone concentration (6.7 nmol/L), in the other ovarihysterectomized ewes progesterone concentrations remained low (<0.9 nmol/L) until surgery (**Paper I**).

The ewes in anoestrus had basal P concentrations that revealed their anoestrous condition. Progesterone treatment increased serum P concentrations in the anoestrous ewes, indicating that the treatments were effective (**Papers II** and **III**). In the study reported in **Paper III**, the length of the luteal phase was determined by measuring P concentrations to verify the expected subnormal or normal luteal phases in GnRH and P + GnRH-treated ewes, respectively; one ewe in each group did not behave as expected and thus were excluded from further analysis. At the time of slaughter (day 5), P concentrations in the GnRH-treated ewes were lower than in the P + GnRH-treated ewes (**Paper III**).

All ewes responded to the GnRH bolus injection with an increase in LH (**Papers II** and **III**) and FSH (**Paper III**) concentrations within 1 h of bolus treatment. Maximum LH concentrations were found 2 h post-bolus injection and the duration of the LH surge was 8 h. Over this time, the LH concentrations remained basal until the time of slaughter in both treated groups (**Papers II** and **III**). While no differences were found in LH concentrations between GnRH- and P + GnRH-treated ewes (**Paper II**), LH concentrations at 1−3 h post-GnRH bolus injection were higher in the GnRH-treated ewes than in the P + GnRH-treated ewes (**Paper III**). From 1 to 5 h post-GnRH bolus injection, FSH concentrations remained high; FSH concentrations at 1 and 5 h post-GnRH bolus injection tended to be higher in the P + GnRH-treated than in the GnRH-treated ewes, P=0.07 (**Paper III**).

The number of small follicles was higher in P-treated than in anoestrous ewes (**Paper II**). The number of small follicles was higher on day 1 than on day 5 after bolus injection in the GnRH-treated ewes (**Paper III**). No differences in the number of medium-sized follicles were found between GnRH- and P + GnRH-treated ewes on day 1 (**Papers II** and **III**) or on day 5 (**Paper III**). The number of

ewes with large follicles one day after GnRH bolus injection was higher in GnRH- than in P + GnRH-treated ewes (**Paper II**). However, no differences in the number of large follicles among the groups were found in **Paper III**. No differences were found in the number of ewes with ruptured follicles (**Papers II** and **III**) or CL (**Paper III**) between GnRH- and P + GnRH-treated ewes. Preovulatory E2 concentrations in GnRH-treated ewes were lower than those found in P + GnRH-treated ewes (**Paper III**). During GnRH treatment, the concentrations of E2 did not increase in ewes treated with GnRH alone, but increased in P + GnRH-treated ewes (**Papers II** and **III**).

Low pituitary ER and PR concentrations were found in anoestrous ewes; in contrast, high receptor concentrations were found in the uteri of the same animals, in spite of the low concentrations of circulating P and E2. Treatment with P did not affect receptor concentrations in the pituitary gland, but decreased the uterine ER and PR concentrations. Treatment with GnRH, either with or without P, increased ten fold pituitary ER and PR concentrations in the anoestrous ewes. GnRH treatment did not increase the uterine steroid receptors concentrations in anoestrous ewes, but it did in the P-pre-treated ewes (**Paper II**).

The presence of pituitary and uterine ERα mRNA was demonstrated in anoestrous ewes (mean ± SEM, amol/μg DNA; 5.12 ± 0.76 and 6.75 ± 0.16 for pituitary gland and uterus respectively). Treatment with P did not affect ERα mRNA concentrations in either the pituitary or the uterus (Figure 1, Tasende *et al.* unpublished data). GnRH treatment (with or without P) increased ERα mRNA concentrations in both the pituitary gland and uterus. The pituitary and uterine ERα mRNA concentrations did not differ between GnRH- and P + GnRH-treated ewes (Figure 1, Tasende *et al.* unpublished data).

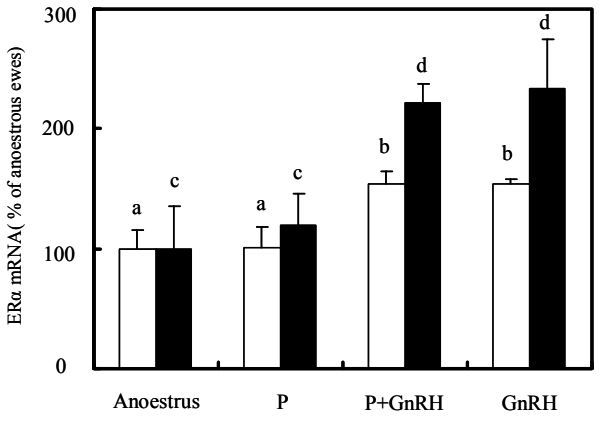

Figure 1. Pituitary □ and uterine ■ ERα mRNA concentrations (mean ± SEM) (presented as percentage of anoestrous ewes) in untreated anoestrous ewes, ewes treated with progesterone (P), GnRH-treated ewes and P + GnRH-treated ewes 1 day after GnRH bolus injection. Bars with different superscripts within the same tissue differs $P < 0.05$. (Tasende *et al*. unpublished data).

The pituitary and uterine steroid receptor concentrations in GnRH- or P + GnRH-treated ewes and cyclic ewes are shown in Figure 2 (Paper III and IV). The main observation pertaining to the data is that GnRH-treated ewes (subnormal luteal phases) presented pituitary and uterine ER and PR patterns that differ from those of P + GnRH-treated (normal luteal phases) and cyclic ewes from day 1 to days 5 or 6. Overall, while steroid receptor profiles in GnRH-treated ewes increased from day 1 to 5, they decreased in P + GnRH-treated and cyclic ewes.

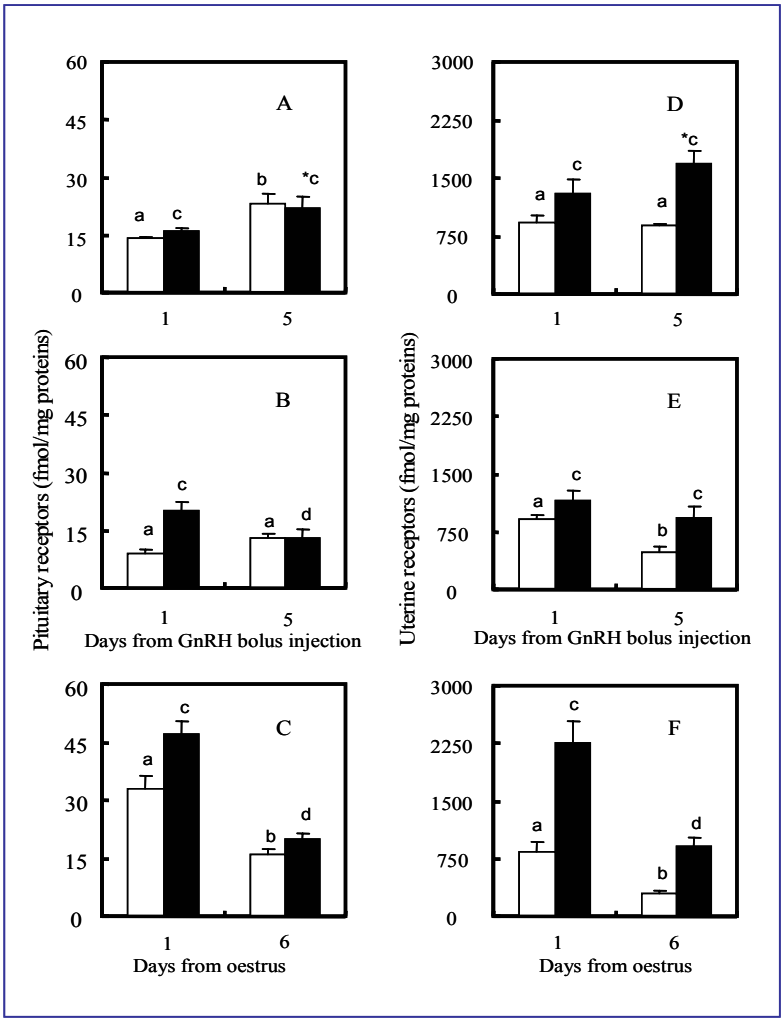

Figure 2. Pituitary (A, B, C) and uterine (D, E, F) concentrations (mean ± SEM) of ER □ and PR ■ in GnRH-treated ewes (A, D) or P + GnRH-treated ewes (B, E) on days 1 or 5 after GnRH bolus injection and in cyclic ewes on days 1 or 6 after oestrus (C, F). In each panel and within the same receptor, bars with different superscripts differ $P < 0.05$; in panels A and D, c vs. *c tendency $P = 0.09$ (**Papers III** and **IV**).

The concentrations of pituitary ER were higher in GnRH-treated than in P + GnRH-treated ewes on both days 1 and 5 ($P<0.03$). Regarding PR, no differences among groups were found on day 1; but on day 5, GnRH-treated ewes had higher PR concentrations than did P + GnRH-treated ewes ($P<0.03$) (Paper IV).

Uterine ER concentrations were no different among the groups on day 1. On day 5, however, the GnRH-treated ewes had higher ER concentrations than did the P + GnRH-treated ewes ($P<0.002$). On day 1, uterine PR concentrations in the GnRH-treated ewes were similar to those of the P + GnRH-treated ewes. On day 5, the GnRH-treated ewes had higher PR concentrations than did the P + GnRH-treated ewes ($P<0.005$) (**Paper III**).

No differences were found in the uterine ERα mRNA concentrations among groups on day 1 after GnRH bolus injection. On day 5, the ERα mRNA concentrations in the GnRH-treated ewes were higher than in the P + GnRH-treated ewes. While the ERα mRNA concentrations in the GnRH-treated ewes increased from day 1 to day 5, ERα mRNA concentrations in the P + GnRH-treated ewes remained similar on all studied days (**Paper III**).

During the normal oestrous cycle, pituitary and uterine ER and PR concentrations on day 1 were higher in both tissues than on days 6 and 13 post oestrus (**Paper IV**) (Figure 2 C and F).

General discussion

Oestrogen and progesterone genomic actions on the target tissues are mediated via their specific nuclear receptors. In addition, receptor affinity needs to be in the range of the circulating concentrations of its hormone. Binding affinity of ER and PR neither differed between different postpartum days (**Paper I**), nor between anoestrous ewes, anoestrous treated ewes or cyclic ewes (**Papers II–IV**). The uterine ER and PR Kd values were similar to those reported in adult (Rexroad, 1981a) and prepubertal ewes (Garófalo & Tasende, 1996; Meikle *et al.,* 1997). The pituitary ER and PR Kd values were also similar to those reported in adult ewes (Wise *et al.*, 1975; Clarke *et al.*, 1981; Glass *et al.*, 1984; Bittman & Blaustein, 1990). The overall results suggest that variations in the responsiveness of the target tissue to these hormones may not depend on changes of affinity, but rather on the binding capacity (receptor concentration).

Low uterine ER and PR concentrations were found in the early postpartum period (days 1 to 5, **Paper I**). This is consistent with the depressed ER and PR concentrations found at parturition, which could be due to the high placental oestrogen and progesterone production (Klauke & Hoffman, 1992). Recently, low ERα expression on day 1 and low PR expression on day 7 postpartum in the glandular epithelium were found (Gray *et al.*, 2003). Similarly, during the early postpartum period the hypothalamic and pituitary ER concentrations were depressed (Wise *et al.*, 1986); this may explain the low circulating gonadotrophin concentrations found in the early postpartum, concentrations responsible for the lack of ovulation (Schirar *et al.*, 1990). During the late postpartum period (days 17 to 30), uterine ER and PR concentrations increased (**Paper I**); as well, cervical ER and PR concentrations were observed to behave similarly in the same ewes (Rodriguez-Piñón *et al.*, 2000). Likewise, the hypothalamic and pituitary ER concentrations (Wise *et al.*, 1986) and the circulating gonadotrophin concentrations (Schirar *et al.*, 1990) were recovered in the late postpartum. These findings are consistent with the follicular status of the ewes in the late postpartum period: all ewes presented large follicles (>4 mm), while only one out of four presented follicles of this size during the early postpartum period. The follicles >4 mm in diameter are oestrogen active (Tsonis *et al* 1984), and the ovarian E2 could up-regulates ER and PR expression (for reviews see Ing *et al.*, 1993; Clark & Many, 1994). Thus, the restoration of uterine ER and PR concentrations during the postpartum period was temporally associated with the presence of oestrogen-active follicles (**Paper I**). Interestingly, only one ewe that had a short luteal phase presented low uterine PR concentrations during the late postpartum period, which is consistent with the known down-regulation exerted by P on its own receptors (Ing *et al.*, 1993; Clark & Mani, 1994).

In our study, 80% of the GnRH-treated and P + GnRH-treated control anoestrous ewes displayed a subnormal or normal luteal phase (**Paper III**). Other researchers report similar results for ewes treated with GnRH alone, while all ewes treated with P + GnRH were found to develop a normal luteal phase (for a review see McLeod *et al.* 1982; McLeod and Haresign, 1984; Southee *et al.* 1988; Hunter, 1991). It was recently reported that the number of ewes with normal CL

among GnRH-treated ewes was similar or lower than among medroxyprogesterone + GnRH-treated ewes, providing GnRH treatment started immediately or one day after progestagen withdrawal, respectively (Bartlewski *et al.*, 2004). The different results obtained by the different researchers may be due to the different schedules of hormone administration and/or the hormone analogue used. Causes of subnormal luteal phases include impaired follicular development, inadequate gonadotrophin secretion and/or premature luteolysis (for reviews, see Hunter, 1991; Garverick *et al*., 1992).

Progesterone treatment increased the number of small follicles in anoestrous ewes (**Paper II**). It has been shown that high P concentrations stimulated follicular turnover, which allowed small follicles to grow (i.e., emergence of a new follicular wave) (Adams *et al.,* 1992; Rubianes, *et al*., 1996). Therefore, we can assume that the GnRH- and P + GnRH-treated ewes had a different population of follicles at the beginning of the GnRH treatment. The number of small follicles decreased from day 1 to day 5 in the GnRH-treated ewes, while remained unchanged in the P + GnRH-treated ewes; these findings are in agreement with those of Ravindra & Rawlings (1997)(**Paper III**). The decrease in the number of small follicles in the GnRH-treated ewes could be explained by a prolongation of the dominance of the largest follicle of wave 1, as low P concentrations prolong the lifespan of the largest follicle, as shown previously by Viñoles *et al.* (1999). Data regarding the number of large follicles one day after bolus injection was contradictory: GnRH-treated ewes had higher (**Paper II**) or similar (**Paper III**) numbers of large follicles than P + GnRH-treated ewes did. Our studies are limited in that observations of follicular status were performed at only one point (the time of slaughter). In agreement with our results, Bartlewski *et al.* (2004), using daily transrectal ultrasonography, found no differences in the numbers of large follicles among GnRH-treated and progestagen + GnRH-treated ewes when GnRH treatment started immediately after progestagen withdrawal. Lower preovulatory E2 concentrations were found in the GnRH-treated than in the P + GnRH-treated ewes (**Paper III**). Similarly, it was found that the E2 concentrations were lower in cows with subnormal luteal phases (Garcia-Winder *et al.*, 1987; Garverick *et al.*, 1988). However, no differences were found in the concentrations of circulating E2 between GnRH-treated ewes with normal and those with subnormal luteal phases (Bartlewski *et al.*, 2001). Differences in ovarian response to GnRH treatment may be due to differences in the gonadotrophic stimuli.

Inadequate gonadotrophin secretion may induce subnormal luteal phases (Hunter, 1991; Garverick *et al*. 1992). The GnRH- and P + GnRH-treated ewes presented FSH and LH surges after the bolus injection (**Paper III**). Simultaneous FSH and LH peaks take place after the onset of oestrus during the breeding season (Pant *et al.* 1977; Campbell *et al.* 1990). The FSH peak tended to be lower in the GnRH-treated than in the P + GnRH-treated ewes (**Paper III**), in agreement with the E2 concentrations found. The timing of the LH surge did not differ among the GnRH-treated ewes, which confirms that the GnRH bolus injection synchronized the onset of the LH surge (**Papers II** and **III**), as shown previously (Hunter *et al.*, 1988). While no differences in LH concentration were found between GnRH-treated and P + GnRH-treated ewes in the study reported in **Paper II**, LH concentrations were, however, found to be higher in the GnRH-treated than P +

GnRH-treated ewes in **Paper III** using a larger number of ewes in the study. Similarly to **Paper III**, Bartlewski *et al.* (2001, 2004) found that the peak concentration of the GnRH-induced LH surge was higher in ewes that developed an inadequate CL than in ewes that developed a normal CL. However, it was reported that the mean concentration, frequency, amplitude and duration of the LH pulses do not differ between cows with short and cows with normal oestrous cycles (Garverick *et al*., 1988). Overall, the data describing LH patterns in animals with short luteal phases are confusing; however, the role of LH can not be discounted, as it plays a key role in ovulation and CL development (Niswender & Nett, 1994). Differences in pituitary sensitivity to ovarian hormones (in terms of, concentrations of steroid receptors) may be involved in the development of subnormal luteal phases.

The pituitary ER and PR concentrations in anoestrous ewes and P-treated ewes were close to the detection limits of the binding assays (**Paper II**) However, the ERα mRNA concentrations in both the pituitary gland and the uterus were similar.. Since PR is considered a marker of oestrogen action (for a review see Clark *et al.,* 1992), the low PR concentrations found are consistent with the low ER concentrations found in the pituitary gland. The low pituitary ER concentrations found in anoestrous ewes do not support the hypothesis that increased sensitivity of the pituitary gland to negative E2 feedback on the frequency of LH pulsatility is due to enhanced ER concentrations (Wise *et al.*, 1975). In contrast to the low pituitary ER and PR concentrations, we found high concentrations of both receptors in the uterus (**Paper II**), in spite of similar ERα mRNA concentrations in both the pituitary gland and the uterus were found (Tasende *et al*., unpublished data), suggesting that the expressions of steroid receptors is regulated by a posttranscriptional mechanism tissue specific. On the other hand, high uterine steroid receptor concentrations together with basal ovarian hormone concentrations were found in anoestrous (**Paper II**).as well as prepubertal ewes (Tasende & Garófalo, 1996; Meikle *et al.,* 1997). While P treatment did not affect the pituitary receptor concentrations, it did decrease the uterine receptor concentrations, as found previously in ovariectomized (Rexroad, 1981b) and prepubertal ewes (Meikle *et al.,* 1997). Nevertheless, P treatment did not affect ERα mRNA concentrations either in the pituitary gland or in the uterus (Tasende *et al*. unpublished data), suggesting that the observed down-regulation effect of P on the uterine receptors is due to a posttranscriptional mechanism (Alarid *et al.*, 1999; Katzenellenbogen., 2000; Preisler-Mashek *et al*., 2002).

GnRH treatment increased pituitary ER and PR concentrations in both anoestrous and P-treated ewes (**Paper II**). Likewise, a direct stimulatory effect of GnRH on pituitary ER concentrations has been demonstrated in the female adult rat (Singh & Muldoon, 1982), the bull calf (Rodriguez & Wise, 1991) and ewes (Clarke *et al*., 2005). On the other hand, the increase in receptor concentrations observed in GnRH-treated ewes could also be due to the concentrations of circulating E2 (**Paper II**). Moreover, crosstalk between the GnRH signal transduction system and gonadotrope ERα has been suggested to be an important modulating mechanism for the E responsiveness of the gonadotrope cell (rat: Demay *et al*., 2001; ewe: Clarke *et al*., 2005).

The increased and similar pituitary ER concentrations found in both GnRH-treated ewes were in agreement with the increased ERα mRNA concentrations found in these ewes on day 1 after bolus injection (Tasende *et al*., unpublished data). It is interesting to note that while the ERα mRNA concentrations increased 2-fold, ER proteins increased 10-fold. The higher steroid receptors concentrations in the pituitary gland at the time of ovulation when compared to the anoestrous season suggest that this increase of sensitivity to the steroid hormones is needed for the pituitary gland to control cyclic function. In contrast to what was found in the pituitary, GnRH treatment increased receptor concentrations in the uterus only in P-treated ewes (anoestrous ewes already had high receptor concentrations). These differences in tissue response suggest that the receptor expression is tissue specific. No differences among ER and PR pituitary concentrations were found among the groups in **Paper II** at the expected time of ovulation (day 1); in contrast, in **Paper IV**, using the same experimental model and a larger number of ewes per group, higher pituitary ER concentrations were found in the GnRH-treated than in the P + GnRH-treated ewes. We have no clear explanation for these contradictory observations; however, preovulatory concentrations of circulating E2 may not be the cause, since E2 concentrations were lower in the GnRH-treated ewes.

The pituitary ER and PR concentrations in anoestrous ewes treated with GnRH, either with or without P priming, were investigated on day 5 after GnRH bolus injection (early luteal phase). Ewes treated with GnRH alone displayed altered dynamics in the pituitary ER and PR pattern, compared to ewes treated with P + GnRH or to cyclic ewes (**Paper IV**). At the time of ovulation of the oestrous cycle, the pituitary ER and PR concentrations were higher than in the luteal phase (**Paper IV**), and similar results for pituitary ER have also been reported (Clarke *et al*., 1981; Tobin *et al.,* 2001). To our knowledge, this is the first report describing pituitary PR concentrations during the oestrous cycle in sheep. Interestingly, pituitary PR concentration followed the same pattern as did pituitary ER concentration; this is consistent with the known up- and down-regulation exerted by E2 and P on the steroid receptor expression at the level of the uterus (for reviews, see Ing *et al*., 1993; Clark & Mani 1994).

It is accepted that early CL development (Days 3−5) in sheep depends on pituitary support (Niswender et al. 2000). During this period, E and P act directly on the pituitary gland to modulate LH secretion, which is required for CL development (Miller et al. 1993, Niswender et al. 2000). In GnRH-treated ewes, pituitary receptor concentrations increased from days 1 to 5 (time of ovulation vs. early luteal phase), while it decreased in P + GnRH-treated and cyclic ewes. The decrease in pituitary PR concentrations observed in the P + GnRH-treated ewes could be due to the down-regulation exerted by the higher concentrations of circulating P found in these ewes in the early luteal phase, similar to those found in the cyclic ewes (**Paper IV**). In contrast, the increased ER and PR concentrations found on day 5 in the GnRH-treated ewes may have been due to lack of P inhibition. At Day 5, higher ER and PR concentrations were found in the pituitary gland of GnRH-treated ewes when compared to P+GnRH-treated ewes. This different pituitary sensitivity to the steroid hormones could be associated with the lifespan of the corpus luteum. A possible explanation could be that at Day 5 a

higher sensitivity of the pituitary gland to P could result in an impaired expression of GnRH receptors and LH synthesis (Miller et al. 1993) resulting in an altered tonic LH secretion and subsequent abnormal function of the CL.

As previously mentioned, the causes of subnormal luteal phases include premature luteolysis, which is due to the premature release of uterine PGF2α (McLeod & Haresign 1984; Hunter *et al.*, 1989). Release of PGF2α is induced by oxytocin (McCracken *et al*., 1999). Oestrogen and P, acting through their corresponding receptors, modulate the release of PGF2α by regulating the concentration of endometrial OxRs (McCracken *et al*., 1999; Goff, 2004). Uterine ER and PR expression at the expected time of premature luteolysis (day 5) differed between GnRH- and P + GnRH-treated ewes (**Paper III**). The decrease observed in the uterine ER concentrations of P + GnRH-treated ewes resembled those found in the early luteal phase of the oestrous cycle (**Paper IV**) and the results reported by Miller *et al.* (1977). However, in the group treated with GnRH alone, the PR concentrations had a tendency to increase from days 1 to 5. The GnRH-treated ewes had higher uterine ER and PR concentrations and lower concentrations of circulating P on day 5 than did the P + GnRH-treated ewes, suggesting that the lower P concentrations were not enough to depress the receptor expression (**Paper III**). In contrast, on day 5 following the first postpartum ovulation, cows expected to have short luteal phases had lower uterine PR concentrations than did cows with normal luteal phases; however, this finding was not associated with differences in the concentrations of circulating P (Zollers *et al.*, 1993). On the other hand, in naturally cyclic ewes, the uterine OxR increase at time of luteolysis may be due to the loss of the inhibitory effect of P (Lau *et al.,* 1993). The low concentrations of circulating P found in the GnRH-treated ewes on day 5 suggest a lack of P dominance which is reflected by the higher uterine receptor expression (compared with the P + GnRH-treated ewes). In addition, uterine ER levels in the GnRH-treated ewes were also higher and it has been reported that oestrogens increase OxR, advancing luteolysis (Wathes and Lamming 1995; McCracken et al. 1999). The induction of steroid receptor expression in the uterus and the hormonal environment found in the GnRH-treated ewes at the expected time of premature luteolysis may be involved in the mechanisms that trigger premature luteolysis.

Conclusions

- The similar affinities of pituitary and uterine oestrogen and progesterone receptors (Kds) found in the different reproductive stages suggest that variations in sensitivity of the target tissue to these hormones may not depend on changes of affinity, but rather on the binding capacity (number of receptors).

- During the postpartum period, the restoration of uterine steroid receptor concentrations is temporally associated with the presence of oestrogen-active follicles on the ovarian surface and with the macroscopic uterine involution.

- In anoestrous ewes treated with GnRH, with or without P, the ER and PR concentrations increased ten fold in the pituitary gland without affecting the uterine receptor concentrations. Progesterone treatment decreased ER and PR receptor concentrations only in the uterus. These results show that regulation of steroid receptor expression by GnRH and P is tissue specific.

- During the oestrous cycle, the pattern of pituitary PR concentration followed that of pituitary ER, being higher at the time of ovulation than in the luteal phase of the oestrous cycle. This is consistent with the known up- and down-regulation exerted by E2 and P on the receptor expression.

- The higher pituitary ER and PR concentrations found in the GnRH-treated ewes, as compared with P + GnRH-treated ewes, reveal differences in the sensitivity of the pituitary gland to steroid hormones, differences that may affect tonic LH secretion.

- The expression of steroid receptors in the uterus and the hormonal environment found in GnRH-treated ewes – when compared to P + GnRH-treated ewes and cyclic ewes – at the expected time of premature luteolysis (day 5) may be involved in the mechanism causing subnormal luteal phases.

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