Regulation of ovine GnRH receptor gene expression by progesterone and oestradiol

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The objective of this study was to determine whether progesterone prevents the stimulatory effects of oestradiol on GnRH receptor gene expression. In Expt 1, ewes were treated during the luteal phase (days 10–12 of the oestrous cycle) with either one or five subcutaneous implants containing oestradiol ($n = 6$ per group). Control ewes received no treatment ($n = 6$). Anterior pituitary glands were collected 16 h after treatment with oestradiol. Steady-state amounts of GnRH receptor mRNA were similar among all three treatment groups despite increased circulating concentrations of oestradiol in implanted ewes at the time of pituitary collection (4.3 ± 0.6 and 24.7 ± 2.6 pg ml⁻¹ in ewes treated with one or five implants, respectively, compared with 0.5 pg ml⁻¹ in controls). Experiment 2 was designed to determine whether progesterone was the ovarian factor preventing the stimulatory effects of oestradiol on expression of the GnRH receptor gene in Expt 1. Twenty-five ewes were ovariectomized on day 6 or day 7 of the oestrous cycle and assigned to one of five treatment groups ($n = 5$ per group). Control ewes received no further treatment. Endogenous luteal phase concentrations of progesterone were replaced in three groups of ewes at the time of ovariectomy via intravaginal implants. Three days after ovariectomy, one group of progesterone-treated ewes received one oestradiol implant, while another group of progesterone-treated ewes received five oestradiol implants. An additional group was treated with five oestradiol implants only, and anterior pituitary glands were collected from all ewes 16 h later. Compared with untreated ovariectomized ewes, treatment with progesterone alone did not affect amounts of GnRH receptor mRNA. In ewes treated with progesterone and either one or five oestradiol implants, steady-state amounts of GnRH receptor mRNA were increased twofold ($P < 0.01$). Treatment with oestradiol in the absence of progesterone increased amounts of GnRH receptor mRNA threefold ($P < 0.001$). These results provide evidence that the stimulatory effects of oestradiol on the expression of the GnRH receptor gene are prevented during the natural luteal phase in ewes. However, progesterone does not appear to act independently to mediate this effect.

Introduction

Oestradiol enhances GnRH-stimulated release of LH from the anterior pituitary gland (Huang and Miller, 1980; Moss and Nett, 1980). One mechanism by which oestradiol increases the sensitivity of the pituitary gland to GnRH is by stimulating expression of the GnRH receptor gene (Sealfon *et al.*, 1990; Wu *et al.*, 1994; Turzillo *et al.*, 1994) which leads to increased numbers of GnRH receptors (Moss *et al.*, 1981; Gregg *et al.*, 1990; Laws *et al.*, 1990b). These effects of oestradiol are likely important during the follicular phase of the reproductive cycle, when increased secretion of oestradiol by developing ovarian

follicles serves to increase the sensitivity of the anterior pituitary gland to GnRH in preparation for the preovulatory surge of LH.

Although oestradiol appears to play a critical role in regulating pituitary responsiveness to GnRH, it may not be the only ovarian steroid involved in regulation of GnRH receptor expression. In cultured ovine pituitary cells, progesterone decreased amounts of GnRH receptor mRNA (Wu *et al.*, 1994) and numbers of GnRH receptors (Laws *et al.*, 1990a). Progesterone also inhibited the oestradiol-induced increase in expression of the GnRH receptor gene *in vitro* (Sealfon *et al.*, 1990; Wu *et al.*, 1994). *In vivo*, high circulating concentrations of progesterone during the luteal phase of the oestrous cycle were associated with a lack of effect of oestradiol on concentrations of GnRH receptor mRNA (Brooks and McNeilly, 1994). Thus, it appears that oestradiol and progesterone have opposing effects on expression of GnRH receptors. It is

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possible that the suppressive effects of progesterone keep the number of GnRH receptors low during the luteal phase. This inhibition is lifted at luteolysis, allowing the stimulatory effects of oestradiol to become dominant.

The purpose of this experiment was to explore the interaction of oestradiol and progesterone on the expression of GnRH receptors in ewes. The first experiment tested the hypothesis that the stimulatory effects of oestradiol on expression of the GnRH receptor gene are prevented during the luteal phase of the oestrous cycle. The two treatments with oestradiol were used to mimic different physiological states. On the basis of the results of Expt 1, Expt 2 was designed to determine whether progesterone is the ovarian factor responsible for preventing the stimulatory effects of oestradiol on expression of the GnRH receptor gene during the luteal phase.

Materials and Methods

All animal experiments were approved by the University of Wyoming Animal Care and Use Committee, and were in accordance with National Institutes of Health (NIH) Guidelines for the use of animals in biological research.

Experiment 1

Eighteen sexually mature western range ewes were housed in outdoor pens and used during the natural breeding season (October). Oestrus was synchronized by administering two i.m. injections of 10 mg prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$, Lutalyse; Upjohn Co., Kalamazoo, MI) 10 days apart. After the second injection of PGF $_{2\alpha}$, ewes were monitored for oestrous behaviour with a vasectomized ram. On day 9 of the oestrous cycle, ewes received either one ($n = 6$) or five ($n = 6$) s.c. implants containing oestradiol (Sigma Chemical Company, St Louis, MO). Implants consisted of Silastic tubing packed with oestradiol as described by Turzillo *et al.* (1995a). Each implant was expected to produce approximately 5 pg oestradiol ml^{-1} in the circulation. Ewes were treated with either one or five oestradiol implants to mimic physiological concentrations during the follicular phase of the oestrous cycle (Karsch *et al.*, 1979; Turzillo *et al.*, 1994) or late pregnancy (Liggins *et al.*, 1973; Carnegie and Robertson, 1978), respectively. Control ewes ($n = 6$) received no implants. Blood samples were collected at intervals of 2 h from the time of treatment with oestradiol until collection of pituitary tissues. In a previous study, increased amounts of pituitary GnRH receptor mRNA were observed 16 h after treatment of ovariectomized ewes with oestradiol implants (Hamernik *et al.*, 1995). Therefore, 16 h after treatment with oestradiol was chosen for collection of pituitary tissues in the present study. Ewes were anaesthetized with sodium thiopental (1 mg kg^{-1} body weight) and exsanguinated. Anterior pituitary glands were hemisected midsagittally, frozen in liquid N_2 and stored at $-70^\circ C$. Serum was harvested from blood samples and stored at $-20^\circ C$.

Experiment 2

Oestrous cycles were induced in 25 sexually mature western range ewes during the anoestrous season (April). Ewes received

one half SynchroMate B progestin implant (Sanofi Animal Health, Inc., Overland Park, KS) s.c. for 12 days to simulate a luteal phase. Upon removal of SynchroMate B, ewes were treated with 500 iu pregnant mares' serum gonadotrophin (PMSG) to induce ovarian follicular development and ovulation. Ewes were monitored for oestrus with a vasectomized ram. On day 6 or day 7 of the induced oestrous cycle, all ewes were bilaterally ovariectomized (OVX) and assigned to one of five treatment groups. Ewes in group 1 received no further treatment and served as OVX controls. Each ewe in groups 2, 3 and 4 ($n = 5$ per group) received one intravaginal implant containing progesterone (Eazi-Breed CIDR; InterAg, Hamilton, NZ) at the time of ovariectomy. These devices are used commercially for synchronization of oestrus in sheep. In a preliminary experiment (data not shown), similar treatment of ovariectomized ewes with CIDR devices for 10 days produced serum progesterone concentrations typical of the luteal phase (> 1 ng ml^{-1}). Three days after ovariectomy and treatment with progesterone, ewes in groups 3 and 4 also received one or five oestradiol implants, respectively, to produce physiological concentrations of serum oestradiol as described above. Ewes in group 5 were not treated with progesterone but received five oestradiol implants 3 days after ovariectomy. Blood samples were obtained from all ewes every 2 h from the time of treatment with oestradiol until collection of pituitary glands 16 h later. Tissues and blood samples were processed and stored as described for Expt 1.

Analysis of mRNA

Polyadenylated RNA was prepared from pituitary tissues (Badley *et al.*, 1988). Northern blot analysis was performed on 2–4 μg of each sample to ensure that RNA was intact, and slot blot analysis was used to quantitate steady-state concentrations of specific mRNAs as described by Turzillo *et al.* (1994). In Expts 1 and 2, concentrations of GnRH receptor mRNA were determined using ovine GnRH receptor cDNA for radio-labelled probe (Turzillo *et al.*, 1994). In Expt 2, concentrations of mRNA encoding LH β subunit were also measured using bovine LH β subunit cDNA for radiolabelled probe (Maurer, 1987). Differences in loading efficiency among RNA samples were adjusted by stripping slot blots of specific cDNA probes and hybridizing to radiolabelled dT (18mer) as described by Turzillo and Nett (1995). Autoradiographs were analysed by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA) for Expt 1 and the NIH 1.52 Image Analysis Program for Expt 2. Concentrations of mRNA encoding GnRH receptor mRNA or LH β subunit are presented as percentages of mean control values for each experiment for consistency and ease of interpretation.

Hormone and receptor assays

Serum concentrations of LH were determined by radioimmunoassay (Niswender *et al.*, 1969) using NIH-oLH-S24 as the reference preparation. The mean limit of detection, intra-assay coefficient of variation (CV) and interassay CV were 80.8 pg ml^{-1} , 7.4% and 6.0%, respectively for two assays. Serum concentrations of oestradiol (Thompson *et al.*, 1978)

were determined by radioimmunoassay. The limit of detection was 0.3 pg ml^{-1} , and the intra- and interassay CVs were $< 10\%$. The concentrations of GnRH receptors in pituitary tissues were determined by a standard curve technique as described by Nett *et al.* (1981).

Statistical analyses

In Expt 1, northern blot analysis revealed that pituitary RNA prepared from one ewe in the group treated with one oestradiol implant was degraded. Therefore, data from this ewe were omitted from statistical analyses. One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS (1987) was used to identify significant effects of treatment. Data describing concentrations of LH and GnRH receptors in Expt 2 were log-transformed before ANOVA to achieve homogeneity of variance. Means were separated using least significant differences. The Pearson correlation coefficient (r) was computed to describe the relationship between concentrations of GnRH receptor mRNA and GnRH receptors in Expt 2. Data are presented as mean $\pm$ SEM.

Results

Experiment 1

Treatment of ewes with one oestradiol implant increased ($P < 0.01$) serum concentrations of oestradiol compared with controls, and treatment with five implants increased concentrations of oestradiol even further ($P < 0.001$; Fig. 1). The mean serum concentrations of LH during the 16 h period of treatment with oestradiol were similar among groups (0.16 ± 0.02 , 0.12 ± 0.01 and $0.12 \pm 0.01 \text{ ng ml}^{-1}$ in ewes treated with zero, one and five oestradiol implants, respectively), and there was no evidence of an LH surge in any animal (data not shown). Amounts of mRNA encoding GnRH receptor were not increased by treatment with oestradiol ($P > 0.09$; Fig. 1).

Experiment 2

Treatment with five oestradiol implants increased serum concentrations of oestradiol ($P < 0.001$; Fig. 2). The mean serum concentration of LH during the 16 h preceding collection of pituitary tissues in control OVX ewes was $1.31 \pm 0.13 \text{ ng ml}^{-1}$. Serum concentrations of LH were similar among ewes treated with progesterone or the combination of progesterone and either one or five oestradiol implants (0.47 ± 0.03 , 0.56 ± 0.11 and $0.34 \pm 0.02 \text{ ng ml}^{-1}$, respectively), but these concentrations were lower ($P < 0.05$) than those in control OVX ewes. In OVX ewes treated only with oestradiol, the mean serum concentration of LH was higher than in any other treatment group ($4.22 \pm 1.31 \text{ ng ml}^{-1}$, $P < 0.05$). In four of five OVX ewes not treated with progesterone, more than tenfold increases in LH were evident either 14 or 16 h after administration of five oestradiol implants, indicating that LH surges were induced in this group (data not shown).

Fig. 1. Mean concentrations of oestradiol (□) in serum and steady-state concentrations of GnRH receptor mRNA (■) in pituitary tissue of ewes during the luteal phase of the oestrous cycle. Control ewes ($n = 6$) received no treatment, while other ewes were treated with one or five implants containing oestradiol (1E or 5E, $n = 5$ or 6 per group, respectively) for 16 h before collection of pituitary glands. Treatment with oestradiol did not alter concentrations of GnRH receptor mRNA.

Treatment of OVX ewes with progesterone did not affect the mean amount of GnRH receptor mRNA relative to that of controls ($P > 0.10$; Fig. 2). Administration of progesterone together with either one or five oestradiol implants increased ($P < 0.01$) concentrations of GnRH receptor mRNA. Ewes treated with five oestradiol implants in the absence of progesterone had higher concentrations of GnRH receptor mRNA relative to both OVX controls ($P < 0.001$) and ewes treated with progesterone and oestradiol ($P < 0.02$). Concentrations of GnRH receptors in pituitary tissue (data not shown) were correlated with amounts of GnRH receptor mRNA ($r = 0.83$, $P < 0.001$).

Concentrations of LH β subunit mRNA are shown (Fig. 3). Concentrations of LH β subunit mRNA were lower ($P < 0.02$) in ewes treated with progesterone, oestradiol, or progesterone and oestradiol together compared with OVX controls.

Discussion

In Expt 1, administration of exogenous oestradiol to ewes during the luteal phase increased serum oestradiol to concentrations comparable with those observed during the follicular phase (Karsch *et al.*, 1979; Turzillo *et al.*, 1994) or late pregnancy (Liggins *et al.*, 1973; Carnegie and Robertson, 1978). However, neither of these physiological concentrations of oestradiol stimulated expression of the GnRH receptor gene during the natural luteal phase of the oestrous cycle. Similarly, Brooks and McNeilly (1994) did not observe a change in amounts of GnRH receptor mRNA 18 h after treatment with $100 \mu\text{g}$ oestradiol during the ovine luteal phase, a dosage which probably resulted in supraphysiological serum concentrations of oestradiol. In contrast, treatment of OVX ewes with oestradiol in Expt 2 resulted in increased concentrations of GnRH receptor mRNA. This effect of oestradiol has been demonstrated previously in OVX ewes (Turzillo *et al.*, 1994; Hamernik *et al.*, 1995; Turzillo and Nett, 1995). Therefore, although oestradiol is a potent stimulator of GnRH receptor gene expression in the absence of endogenous ovarian

Fig. 2. Mean concentrations of oestradiol in serum (□) and steady-state concentrations of GnRH receptor mRNA (■) in pituitary tissue of ovariectomized (OVX) ewes ($n = 5$ per group). Progesterone (P) was replaced at the time of OVX in three groups of ewes via intravaginal implants. Three days after OVX, three groups of ewes were treated with either one or five implants containing oestradiol (1E or 5E, respectively) for 16 h before collection of pituitary glands. Significant differences among mean concentrations of GnRH receptor mRNA ($P < 0.02$) are indicated by different letters.

Fig. 3. Mean concentrations of LHβ subunit mRNA in pituitary tissue of ovariectomized (OVX) ewes ($n = 5$ per group). Progesterone (P) was replaced at the time of OVX in three groups of ewes by intravaginal implants. Three days after OVX, three groups of ewes were treated with either one or five implants containing oestradiol (1E or 5E, respectively) for 16 h before collection of pituitary glands. Significant differences among mean concentrations of GnRH receptor mRNA ($P < 0.02$) are indicated by different letters.

influences, this stimulatory effect is prevented during the luteal phase *in vivo*.

During the luteal phase in sheep, progesterone secreted by the corpus luteum is the major circulating hormone of ovarian origin. Since treatment of cultured ovine pituitary cells with progesterone prevents the stimulatory effects of oestradiol on ovine GnRH receptor gene expression (Sealfon *et al.*, 1990; Wu *et al.*, 1994), the present study tested the hypothesis that progesterone is the endocrine factor responsible for preventing oestradiol-induced stimulation of GnRH receptor gene expression during the luteal phase *in vivo*. In contrast to expectations based on results obtained *in vitro*, treatment with oestradiol

caused a twofold increase in concentrations of mRNA encoding GnRH receptor and a 3.5-fold increase in the number of GnRH receptors in ewes pretreated with progesterone. These results are in agreement with those of Schoenemann *et al.* (1985), who reported that treatment of ovariectomized cows with progesterone for 7 days did not prevent oestrogen-induced increases in pituitary concentrations of GnRH receptors. Reasons for the contradictory effects of progesterone on expression of GnRH receptors *in vitro* and *in vivo* are not clear. However, the evidence obtained *in vivo* indicates that progesterone is not the only ovarian hormone with inhibitory effects on GnRH receptor expression in ruminants. In a previous study, increased concentrations of mRNA encoding GnRH receptor were observed 12 h after removal of the corpus luteum in ewes (Turzillo and Nett, 1995). This increase occurred in the absence of increases in serum concentrations of oestradiol. These results led us to postulate that removal of progesterone was essential for enhanced expression of the GnRH receptor gene. However, in light of the inability of progesterone to oppose effects of oestradiol on GnRH receptors in cattle and sheep, it is possible to speculate that another endocrine factor of luteal origin may work together with progesterone to suppress expression of GnRH receptors during the luteal phase of the oestrous cycle. The identity of such a factor is unknown at this time.

Although ovary-intact ewes were not included in Expt 2, it has been shown that both concentrations of GnRH receptor mRNA and numbers of GnRH receptors remain unchanged 12–16 h after ovariectomy (Hamernik *et al.*, 1995; Turzillo and Nett, 1995). Therefore, it is assumed that expression of the GnRH receptor gene did not change after ovariectomy in the present study. Replacement of progesterone in ovariectomized ewes without subsequent treatment with oestradiol did not affect concentrations of GnRH receptor mRNA. The lack of difference in concentrations of GnRH receptor mRNA between OVX and OVX–progesterone-treated ewes does not support the results of studies conducted *in vitro*, in which progesterone

decreased expression of the GnRH receptor gene (Wu *et al.*, 1994) and binding of GnRH to ovine pituitary cells (Laws *et al.*, 1990a). Moreover, an effect of progesterone on GnRH receptor gene expression was not demonstrated in cultured anterior pituitary cells collected from ewes during the luteal or follicular phase of the oestrous cycle (A. M. Turzillo and T. M. Nett, unpublished). While reasons for these discrepancies are unclear, the results do not provide evidence for a direct pituitary action of progesterone alone on expression of the ovine GnRH receptor.

Increases in GnRH receptor mRNA induced by treatment with oestradiol in the presence of progesterone were of lower magnitude than the increase induced by oestradiol in the absence of progesterone (Fig. 2). This finding indicates that, while progesterone was not completely effective in opposing the stimulatory effects of oestradiol, it did prevent maximal expression of the GnRH receptor gene. These results are in partial agreement with previous reports (Sealfon *et al.*, 1990; Wu *et al.*, 1994) that addition of progesterone to ovine pituitary cultures attenuated oestradiol-stimulated increases in concentrations of GnRH receptor mRNA and GnRH receptors. However, *in vivo*, additional influences of hypothalamic GnRH must also be considered. Both oestradiol and progesterone exert negative feedback effects on GnRH secretion (Karsch *et al.*, 1987). In the absence of progesterone, oestradiol can act acutely as a positive regulator of GnRH secretion resulting in GnRH and LH surges (Moenter *et al.*, 1990). Although the schedule of blood sampling in the present study did not permit precise characterization of LH (or GnRH) pulse frequency, higher mean concentrations of LH and the occurrence of LH surges indicate that GnRH secretion during the 16 h before pituitary collection was greater in ewes treated only with oestradiol compared with ewes treated with both oestradiol and progesterone. Since GnRH stimulates expression of its own receptor (Nett *et al.*, 1981; Khalid *et al.*, 1987; Turzillo *et al.*, 1995b), increased GnRH together with oestradiol probably resulted in higher concentrations of GnRH receptor mRNA in ewes treated only with oestradiol compared with ewes exposed to progesterone before treatment with oestradiol.

Ovariectomy increases the amounts of mRNA encoding gonadotropin subunits, including the LH β subunit (Nilson *et al.*, 1983). In Expt 2, concentrations of LH β subunit mRNA in OVX ewes treated with progesterone or oestradiol, or both, were lower than in untreated OVX ewes. These findings indicate that the castration-induced increase in expression of the LH β subunit gene can be prevented by immediate replacement with progesterone, and can be reversed by treatment for 16 h with oestradiol (3 days after ovariectomy) in the absence of progesterone. Negative feedback effects of oestradiol on amounts of ovine LH β subunit mRNA *in vivo* have been documented previously (Nilson *et al.*, 1983; Landefeld *et al.*, 1984; Herring *et al.*, 1991) and involve direct actions at the anterior pituitary gland (DiGregorio and Nett, 1995). The results of the present study indicate that progesterone also regulates expression of the ovine LH β subunit gene; this effect is probably mediated indirectly via negative feedback effects of progesterone on the hypothalamic secretion of GnRH (Karsch *et al.*, 1987).

In summary, treatment of ewes with physiological concentrations of oestradiol during the luteal phase of the oestrous

cycle did not elicit significant changes in expression of the GnRH receptor gene. Progesterone alone does not appear to inhibit the effects of oestradiol during the luteal phase, since replacement of progesterone in ovariectomized ewes did not prevent oestradiol-induced increases in concentrations of GnRH receptor mRNA. Further investigation is necessary to identify the ovarian factor(s) that inhibits stimulation of GnRH receptor gene expression in response to oestradiol during the luteal phase.

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