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Effects of estradiol on concentrations of gonadotropin-releasing hormone receptor messenger ribonucleic acid following removal of progesterone

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To test the hypothesis that low levels of estradiol are sufficient to increase concentrations of GnRH receptor mRNA in the absence of progesterone, ewes were ovariectomized and immediately treated with estradiol implants for 12 h to achieve circulating concentrations of estradiol typical of the early ($n = 5$) or late ($n = 4$) follicular phase. Five additional ewes underwent lutectomy, and control ewes were untreated. Treatment of ewes with ½ or 1 estradiol implant increased concentrations of estradiol in serum to 3.0 ± 0.8 pg/ml or 6.3 ± 0.3 pg/ml, respectively, and concentrations of estradiol in lutectomized ewes (2.4 ± 0.5 pg/ml) were intermediate. Ovariectomy did not alter concentrations of GnRH receptor mRNA or numbers of GnRH receptors. Treatment of ewes with 1 estradiol implant increased concentrations of GnRH receptor mRNA and numbers of GnRH receptors. In ewes treated with ½ estradiol implant, concentrations of GnRH receptor mRNA were intermediate between controls and ewes treated with 1 estradiol implant, and numbers of GnRH receptors were greater than controls. Lutectomy increased concentrations of GnRH receptor mRNA but did not affect numbers of GnRH receptors. We conclude that estradiol stimulates expression of the GnRH receptor gene and numbers of GnRH receptors in the absence of progesterone. However, effects of estradiol on expression of the GnRH receptor gene were clearly evident only when concentrations of estradiol were elevated to levels typical of the late follicular phase.

Keywords: GnRH receptor; estradiol; sheep

Introduction

Synthesis and secretion of gonadotropic hormones are stimulated following binding of hypothalamic GnRH to specific receptors in the anterior pituitary gland. Therefore, regulation of GnRH receptors represents an important component in the coordination of events that lead to reproductive cyclicity in females. Expression of the GnRH receptor gene and numbers of GnRH receptors are influenced by ovarian steroids. In the ewe, steady-state concentrations of GnRH receptor mRNA (Turzillo *et al.*, 1994; Hamernik *et al.*, 1995) and numbers of GnRH receptors (Moss *et al.*, 1981; Gregg & Nett, 1989) were increased by treatment with estradiol. In contrast, progesterone appears to have inhibitory effects on ovine GnRH receptors. In cultured ovine anterior pituitary cells, amounts of GnRH receptor mRNA (Wu *et al.*, 1994) and numbers of GnRH receptors (Laws *et al.*, 1990a) were decreased by treatment with progesterone, and stimulation of concentrations of GnRH receptor mRNA by estradiol were attenuated by progesterone (Sealfon *et al.*, 1990). Recently it was reported that treatment of ewes with estradiol does not affect GnRH receptor gene expression during the luteal phase of the estrous cycle, when concentrations of progesterone are high (Brooks & McNeilly, 1994). Potentially opposing effects of estradiol and progesterone are physiologically relevant to the preovulatory period, when changes in circulating levels of these hormones may regulate increases in GnRH receptor gene expression and numbers of GnRH receptors prior to the ovulatory LH surge (Crowder & Nett, 1984; Brooks *et al.*, 1993; Turzillo *et al.*, 1994).

We previously observed increased steady-state concentrations of GnRH receptor mRNA in association with decreased concentrations of circulating progesterone during luteolysis (Turzillo *et al.*, 1994). This change in GnRH receptor gene expression occurred early in the preovulatory period, prior to an increase in circulating concentrations of estradiol. Based on these observations, we postulated that increased expression of the ovine GnRH receptor gene at this time is initiated by removal of inhibitory effects of progesterone and occurs independent of changes in circulating concentrations of estradiol. Subsequently, it was reported by Hamernik *et al.* (1995) that removal of ovarian steroids for 16 h by ovariectomy failed to affect concentrations of GnRH receptor mRNA in ewes, thus indicating that the continued influence of an ovarian hormone(s) may be required to stimulate GnRH receptor gene expression while concentrations of progesterone are decreasing. Although increased concentrations of circulating estradiol are not requisite for inreased GnRH receptor gene expression, it is possible that low levels of estradiol in serum, similar to those observed during the early follicular phase, may have a stimulatory effect on GnRH receptor gene expression following removal of inhibitory effects of progesterone. Therefore, the present study was designed to test the hypothesis that low levels of estradiol in the ewe are sufficient to increase concentrations of GnRH receptor mRNA in the absence of progesterone.

Results

Before treatments were initiated on day 11 or 12 of the estrous cycle, mean serum concentrations of estradiol and progesterone were 1.4 ± 0.2 pg/ml and 2.73 ± 0.2 ng/ml, respectively ($n = 24$ ewes), and there were no differences among treatment groups ($P > 0.55$). Circulating concentrations of estradiol and progesterone at the time of pituitary collection are illustrated in Figure 1. Ovariectomy (OVX) did not affect mean concentrations of estradiol in serum. Compared to controls, concentrations of estradiol were elevated ($P < 0.05$) in ewes that received ½ estradiol implant, and concentrations of estradiol were elevated further in ewes treated with 1 estradiol implant ($P < 0.01$ compared to ½ estradiol implant). Concentrations of estradiol in lutectomized ewes were intermediate between levels in controls and ewes treated with ½ estradiol implant. Mean circulating concentrations of progesterone decreased following OVX and lutectomy (LUTX) and were >87% lower ($P < 0.01$) than levels in controls in all other groups at the time of pituitary collection.

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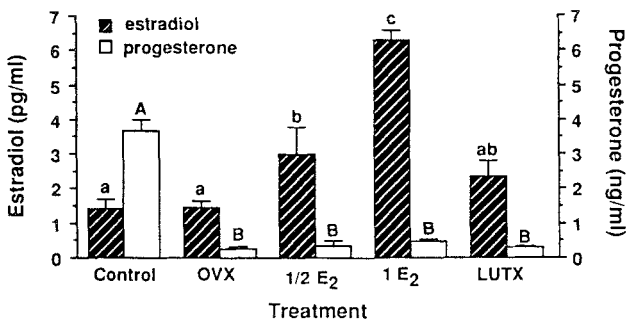

Figure 1 Concentrations (mean ± SEM) of estradiol and progesterone in serum collected from control ewes ($n = 5$), ovariectomized ewes (OVX; $n = 5$), OVX ewes treated with ½ estradiol implant (½ E; $n = 5$), OVX ewes treated with 1 estradiol implant (1 E; $n = 4$) and lutectomized ewes (LUTX; $n = 5$). Significant differences ($P < 0.05$) among means are indicated by lowercase letters for estradiol and uppercase letters for progesterone

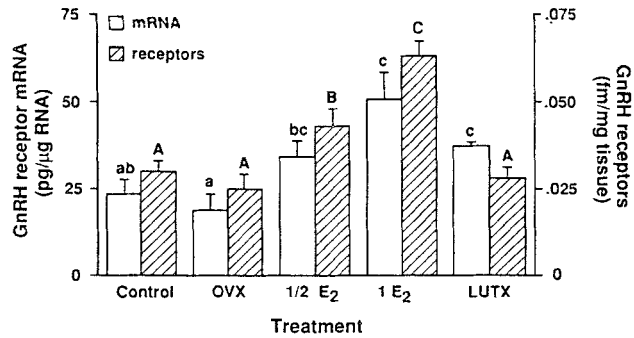

Figure 2 Concentrations (mean ± SEM) of GnRH receptor mRNA and GnRH receptors in pituitary glands of control ewes ($n = 5$), ovariectomized ewes (OVX; $n = 5$), OVX ewes treated with ½ estradiol implant (½ E; $n = 5$), OVX ewes treated with 1 estradiol implant (1 E; $n = 4$) and lutectomized ewes (LUTX; $n = 5$). Significant differences ($P < 0.05$) among means are indicated by lowercase letters for concentrations of GnRH receptor mRNA and uppercase letters for concentrations of GnRH receptors

The mean number of LH pulses during the 12 h sampling period was 1.6 ± 0.4 in control ewes. Ovariectomy and LUTX increased ($P < 0.001$) the frequency of LH pulses to 9.2 ± 1.7 and 8.6 ± 1.0 in 12 h, respectively. Mean numbers of LH pulses were similar between ewes treated with ½ estradiol implant (5.6 ± 1.6 in 12 h) and ewes treated with 1 estradiol implant (5.3 ± 1.3 in 12 h), and were less ($P < 0.01$) in estradiol-implanted ewes than in OVX and LUTX ewes.

Twelve h following OVX, mean steady-state concentrations of GnRH receptor mRNA were not different from controls (Figure 2). Concentration of GnRH receptor mRNA were higher ($P < 0.01$) in ewes that received 1 estradiol implant than in controls. In ewes treated with ½ estradiol inplant, concentrations of GnRH receptor mRNA were intermediate between controls and ewes that received 1 estradiol implant ($P = 0.11$ compared to controls, Figure 2). LUTX increased ($P < 0.05$) concentrations of GnRH receptor mRNA compared to controls.

Mean numbers of GnRH receptors were similar between control, OVX and LUTX ewes (Figure 2). Treatment with ½ estradiol implant increased ($P < 0.05$) numbers of GnRH receptors compared with controls. Numbers of GnRH receptors were higher ($P < 0.01$) following treatment with 1 estradiol implant than after treatment with ½ estradiol implant.

Discussion

The endocrine regulation of GnRH receptors most likely involves a precisely coordinated interplay of hypothalamic, ovarian and possibly hypophyseal inputs. The present study focused on the importance of the ovarian steroids estradiol and progesterone in regulating GnRH receptors. Removal of the ovaries resulted in decreased serum concentrations of progesterone but did not affect concentrations of GnRH receptor mRNA or numbers of GnRH receptors. These findings confirm previous results (Hamernik *et al.*, 1995) and indicate that following withdrawal of progesterone, another hormone(s) of ovarian origin may be required to stimulate expression of GnRH receptors. Replacement of endogenous estradiol with 1 estradiol implant resulted in circulating concentrations of estradiol similar to those observed during the late follicular phase (Karsch *et al.*, 1979; Karsch *et al.*, 1980; Turzillo *et al.*, 1994) and increased concentrations of GnRH receptor mRNA and numbers of GnRH receptors. These results confirm those of previous studies demonstrating the stimulatory effects of estradiol on GnRH receptors *in vivo* (Moss *et al.*, 1981; Gregg & Nett, 1989; Turzillo *et al.*, 1994) and provide further evidence that estradiol is likely important for stimulating maximal expression of GnRH receptors during the later stages of the preovulatory period. However, replacement of circulating levels of estradiol typical of the early follicular phase (½ estradiol implant) did not increase GnRH receptor gene expression significantly but resulted in concentrations of GnRH receptor mRNA intermediate between controls and ewes with peripheral levels of estradiol typical of the late follicular phase (1 estradiol implant). Therefore, estradiol may not be the sole factor involved in increasing GnRH receptor gene expression during the early preovulatory period. In LUTX ewes, the endogenous source of progesterone was removed and the endogenous source of estradiol and the remainder of the ovaries was left intact. Despite similar peripheral concentrations of estradiol, a greater and more consistent increase in concentrations of GnRH receptor mRNA was observed in LUTX ewes than in ewes treated with ½ estradiol implant. Increased concentrations of GnRH receptor mRNA in LUTX ewes were not accompanied by increased numbers of GnRH receptors, and this finding is similar to those of a previous study (Turzillo *et al.*, 1994) in which increased concentrations of GnRH receptor mRNA occurred 12 h following $PGF_{2\alpha}$-induced luteolysis but increased numbers of GnRH receptors were first noted at 24 h. It is possible that in LUTX ewes, an ovarian hormone other than estradiol contributed to the elevation in concentrations of GnRH receptor mRNA. This idea is supported by previous observations in ovary-intact ewes in which concentrations of GnRH receptor mRNA increased following induction of luteolysis with $PGF_{2\alpha}$ but prior to an increase in circulating concentrations of estradiol (Turzillo *et al.*, 1994). Inhibin has been shown to increase concentrations of GnRH receptor mRNA in cultured ovine pituitary cells (Sealfon *et al.*, 1990), and stimulatory effects of estradiol and inhibin on numbers of GnRH receptors are additive (Gregg *et al.*, 1991; Wu *et al.*, 1994). Since there is evidence that circulating levels of inhibin increase following $PGF_{2\alpha}$-induced luteolysis (Findlay *et al.*, 1990), it is intriguing to speculate that inhibin of follicular origin together with endogenous estradiol stimulated expression of the GnRH receptor gene in LUTX ewes. Further experiments are warranted to determine whether inhibin regulates GnRH receptors *in vivo*.

Despite the lack of significant change in concentrations of GnRH receptor mRNA, numbers of GnRH receptors were increased in ewes treated with ½ estradiol implant. In previous studies, increased steady-state levels of GnRH receptor mRNA either preceded (Brooks *et al.*, 1993; Turzillo *et al.*, 1994) or accompanied (Brooks & NcNeilly, 1994; Turzillo *et al.*, 1995b) increases in numbers of GnRH receptors. The discordance between concentration of GnRH recep-

tor mRNA and numbers of GnRH receptors following replacement with ½ estradiol implant in the present study may be related to the duration of exposure to estradiol. During the ovine estrous cycle, circulating concentrations of estradiol rise gradually during the follicular phase. In contrast, concentrations of estradiol in ovariectomized ewes in this experiment reached maximal levels within 4 h of placement of implants and remained steady thereafter (data not shown). Therefore, this treatment regime did not precisely mimic changes that occur during the natural follicular phase. In ovine anterior pituitary cells, numbers of GnRH receptors increased after 8 h of exposure to estradiol and were maximal after 12 h (Gregg *et al.*, 1990). The temporal relationship between concentrations of GnRH receptor mRNA and numbers of GnRH receptors during the first 12 h following treatment with estradiol has not been characterized; however, if the response to low doses of estradiol *in vivo* is similar to the response *in vitro*, then maximal increases in expression of the GnRH receptor gene in ewes treated with ½ estradiol implant may have occurred sooner than during the natural follicular phase and before pituitary glands were collected at 12 h. Higher levels of estradiol in ewes treated with ≥ 1 estradiol implant (Hamernik *et al.*, 1995; present study; Turzillo *et al.*, 1995a) may provide a more potent stimulation of GnRH receptor gene expression, resulting in more pronounced elevations of concentrations of GnRH receptor mRNA concomitant with increased numbers of GnRH receptors after 12–16 h. Alternatively, it is possible that exogenous estradiol, in the absence of other ovarian influences, may have up-regulated numbers of GnRH receptors in ewes that received ½ implant by increasing translational efficiency of GnRH receptor mRNA (which has been observed in gonadotrope-derived αT3-1 cells; Tsutsumi *et al.*, 1993) and/or recycling of preexisting receptors (Svartz & Hazum, 1987).

Results of the present study taken together with previous findings indicate that the stimulatory effects of estradiol on ovine GnRH receptors may be manifested only when progesterone is absent. In most experiments, positive effects of estradiol on concentrations GnRH receptor mRNA and/or numbers of GnRH receptors have been observed either in cultured anterior pituitary cells (Gregg *et al.*, 1990; Laws *et al.*, 1990b; Sealfon *et al.*, 1990) or in ovariectomized ewes (Moss *et al.*, 1981; Turzillo *et al.*, 1994; Hamernik *et al.*, 1995). In contrast, treatment of luteal phase ewes with estradiol failed to elicit increases in GnRH receptor mRNA or numbers of GnRH receptors (Brooks & McNeilly, 1994), and progesterone attenuates effects of estradiol *in vitro* (Sealfon *et al.*, 1990). These observations support the idea that during the ovine estrous cycle, GnRH receptors are regulated in part by opposing effects of progesterone and estradiol.

Recently we reported that increasing GnRH pulse frequency for 12 h led to increased concentrations of GnRH receptor mRNA in ewes (Turzillo *et al.*, 1995b). These results together with evidence that GnRH also increases numbers of GnRH receptors (Nett *et al.*, 1981; Khalid *et al.*, 1987) led to the hypothesis that increased concentrations of GnRH receptor mRNA during the preovulatory period may be mediated in part by increased secretion of GnRH following removal of negative feedback effects of progesterone. Results of the present study provide further insight into the role of GnRH in regulating expression of the GnRH receptor gene. As anticipated, both OVX and LUTX resulted in dramatic increases in GnRH pulse frequency, as evidenced by LH pulse frequency, due to removal of ovarian negative feedback (primarily that of progesterone; Karsch, 1987). Although LH pulse frequency was similar between OVX and LUTX ewes, concentrations of GnRH receptor mRNA increased only in response to LUTX. Therefore, although increased frequency of GnRH pulses may be important in stimulating GnRH receptor gene expression following removal of progesterone, it appears that this stimulatory effect requires the presence of the ovary. The exact mechanism by which ovarian hormone(s) may modulate the effects of GnRH on concentrations of GnRH receptor mRNA requires further investigation.

In summary, when circulating concentrations of estradiol were elevated following OVX to levels typical of the late follicular phase, concentrations of GnRH receptor mRNA and numbers of GnRH receptors increased. However, in the presence of peripheral concentrations of estradiol typical of the early follicular phase, concentrations of GnRH receptor mRNA were intermediate between levels in control luteal phase ewes and ewes exposed to higher concentrations of estradiol. Removal of endogenous progesterone by LUTX increased concentrations of GnRH receptor mRNA in the presence of endogenous levels of estradiol similar to those of the early follicular phase. These results indicate that estradiol stimulates expression of the GnRH receptor gene in the absence of progesterone, and may contribute to increased concentrations of GnRH receptor mRNA during the early preovulatory period. However, estradiol may not be the sole regulator of GnRH receptor gene expression during the early follicular phase.

Materials and methods

Animals and treatments

Twenty-five sexually mature western range ewes were used in this experiment during the natural breeding season in Colorado. All procedures were approved by the Colorado State University Animal Care and Use Committee and were in accordance with NIH guidelines for the use of animals in biological research.

Implants were prepared by packing Silastic tubing with crystalline estradiol-17β (estradiol; Sigma Chemical Co., St. Louis, MO) as described previously (Turzillo *et al.*, 1995a). Each full-size implant (2.6 mm i.d., 4.8 mm o.d., 5 cm total length) was designed to produce approximately 5 pg/ml estradiol in the circulation (Karsch *et al.*, 1980). Treatment of individual ewes with ½ implant was expected to produce concentrations of estradiol similar to those observed during the early follicular phase (0–24 h following the initial decrease in concentrations of progesterone, 3–4 pg/ml; Karsch *et al.*, 1979; Karsch *et al.*, 1980; Turzillo *et al.*, 1994), while 1 implant was expected to elevate estradiol to levels normally observed during the late follicular phase (24–48 h following the initial decrease in progesterone, 5–7 pg/ml; Karsch *et al.*, 1979; Karsch *et al.*, 1980). On day 11 or 12 of the estrous cycle, 15 ewes were bilaterally ovariectomized (OVX) and immediately received ½ estradiol implant (n = five ewes), 1 estradiol implant (n = five ewes), or no implant (n = five ewes). Implants were placed subcutaneously in the axillary region. To remove the endogenous source of progesterone while leaving the remainder of the ovaries intact, five ewes underwent lutectomy (removal of all corpora lutea present, LUTX) on day 11 or 12 of the estrous cycle. An additional five untreated, ovary-intact ewes served as controls.

Blood samples were obtained prior to treatments (OVX or LUTX) and at 15 min intervals for 12 h following initiation of treatments. Sera were harvested and stored at −20°C. Pituitary glands were collected following anesthesia with sodium pentobarbital and exsanguination, hemisected, midsagitally, frozen immediately on dry ice, and stored at −80°C.

Analysis of mRNA

Polyadenylated (poly(A)$^+$)-RNA was prepared from pituitary tissues (Badley *et al.*, 1988). The integrity of each RNA sample was verified by Northern blot analysis. Two μg poly(A)$^+$ RNA were applied to nylon filters (Hybond; Amersham, Arlington Heights, IL) using a slot blot apparatus.

Blots were probed with radiolabeled ovine GnRH receptor cDNA as previously described (Turzillo *et al.*, 1994). A standard curve was generated by including duplicate aliquots of varying amounts (6.25, 12.5, 25, 50, 100, 200, 400 pg) of sense GnRH receptor RNA on the nylon filters. Autoradiographs were analysed using the NIH 1.52 Image Analysis Program. To normalize unequal loading of RNA samples, filters were stripped of GnRH receptor cDNA as previously described (Turzillo *et al.*, 1994) and re-probed with dT (18mer) which was end-labeled with [^{32}P]γATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Hybridization to radiolabeled dT was performed at 30°C for 1 h in 5 × SSC, 10 mM $NaHPO_4$, 1 mM NaH_2PO_7 and 0.12% sarkosyl. Filters were washed in 2 × SSC at 30°C and exposed to film for 20 min (Juengel *et al.*, 1994). After normalization to relative amounts of dT, steady-state concentrations of GnRH receptor mRNA were expressed as pg/μg poly(A)$^+$ RNA.

Hormone and receptor assays

Concentrations of estradiol in sera were determined in a single RIA (Thompson *et al.*, 1978). The limit of detection was 1.1 pg/ml estradiol and the intra-assay coefficient of variation (CV) was 7.0%. Concentrations of progesterone in sera (Niswender, 1973) were determined in two assays. The mean limit of detection was 85 pg/ml progesterone, intra-assay CV was 6.7%, and inter-assay CV was 12.1%. Concentrations of LH in sera (Niswender *et al.*, 1969) were determined in three assays uing NIH-oLH-S24 as the standard preparation. The mean limit of detection was 85 pg/ml, intra-assay CV was 8.3%, and inter-assay CV was 14.8%.

Concentrations of GnRH receptors were quantified by binding of [^{125}I]-D-Ala^6-GnRH-EA to an enriched membrane fraction prepared from each pituitary sample (Nett *et al.*, 1981) as described previously (Turzillo *et al.*, 1995b).

Statistical analyses

Serum concentrations of estradiol in one ewe treated with 1 estradiol implant reached extremely high levels due to a crack in the wall of the implant; data from this ewe were eliminated from the study. Effects of treatments were analysed by 1-way ANOVA using the general linear model procedure of SAS (SAS, 1987). When heterogeneity of variance was detected, data were log-transformed. Differences among treatment groups in mean concentration of GnRH receptor mRNA, numbers of GnRH receptors, and circulating concentrations of estradiol and progesterone were assessed by least significant differences (SAS, 1987). Pulses of LH were identified as two sequential samples with non-overlapping confidence intervals (Duddleson *et al.*, 1972). Differences among treatment groups in frequency of LH pulses were evaluated by Chi-square analysis. All data are presented as mean ± SEM.

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