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Estradiol and Gonadotropin-Releasing Hormone (GnRH) Interact to Increase GnRH Receptor Expression in Ovariectomized Ewes After Hypothalamic–Pituitary Disconnection

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Gonadotropin-releasing hormone (GnRH) receptor expression is regulated by estradiol and GnRH itself. The objective of this experiment was to determine the extent to which low levels of estradiol, similar to those observed during the transition from the luteal to the follicular phase of the estrous cycle, and GnRH interact to regulate expression of GnRH receptors and GnRH receptor mRNA. Ewes were ovariectomized (OVX) at least 2 wk prior to initiation of the experiment, and the pituitary gland was surgically disconnected from the hypothalamus to remove ovarian and hypothalamic inputs to the pituitary. Within 24 h after hypothalamic–pituitary disconnection, ewes received pulses of GnRH (250 ng/pulse) every 2 h for 6 d. At the end of 6 d, ewes were randomly assigned to treatments in a 2 × 2 factorial arrangement as follows: half of the animals received a single estradiol implant and half received an empty implant (placebo). At the same time, animals also received one of the following treatments: (1) saline or (2) GnRH (100 ng/pulse/2 h). Additionally, one group of ewes was ovariectomized, but not subjected to hypothalamic–pituitary disconnection (OVX controls). Blood samples were collected 15 min prior to each pulse of GnRH or saline and at 15-min intervals for 1 h after each pulse until tissues were collected and concentrations of luteinizing hormone (LH) were determined. Anterior pituitaries were collected 24 h after implant insertion to quantitate steady-state amounts of GnRH receptor mRNA and numbers of GnRH receptors. Mean LH was greatest in ovariectomized control ewes compared to all other treatments ($p < 0.05$). Mean LH and LH pulse amplitude in the placebo and GnRH-treated group most closely mimicked LH secretion in ovariectomized control animals. Mean LH and LH pulse amplitude were similar between both GnRH-treated groups ($p < 0.05$). Mean LH and LH pulse amplitude were significantly lower in all animals treated with saline compared to OVX controls ($p < 0.05$). Treatment with an estradiol implant and pulsatile GnRH increased ($p < 0.05$) relative amounts of GnRH receptor mRNA and the number of GnRH receptors compared to all other treatments. There were no differences in GnRH receptor expression between the remaining treatment groups ($p > 0.05$). Therefore, in OVX ewes after hypothalamic–pituitary disconnection, low levels of estradiol and GnRH are required to increase GnRH receptor mRNA and GnRH receptor numbers. Since we only observed an increase in GnRH receptor expression in the presence of both estradiol and GnRH, we conclude that there is a synergistic interaction between these two hormones in the regulation of GnRH receptor expression.

Key Words: Estradiol; GnRH; GnRH receptors; mRNA; sheep.

Introduction

The number of gonadotropin-releasing hormone (GnRH) receptors increases within 24 h of prostaglandin $F_2\alpha$-induced luteolysis, remains elevated during the preovulatory luteinizing hormone (LH) surge, and declines after the surge to luteal phase levels *(1)*. Increased numbers of GnRH receptors during the follicular phase of the estrous cycle suggests that regulation of the number of GnRH receptors may be necessary for the induction of the preovulatory LH surge.

Estradiol and GnRH appear to be the primary stimulators of GnRH receptor expression during the follicular phase in ewes; however, the relative importance of GnRH or estradiol is not clear. Several studies provided evidence that increased expression of GnRH receptors in response to

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estradiol is mediated directly at the level of the pituitary. Injection of estradiol (100 μg) into ovariectomized ewes that had received desensensitizing infusions of GnRH resulted in an increased number of GnRH receptors and provided early evidence that estradiol exerts its actions directly at the level of the pituitary *(2)*. Estradiol increased the number of receptors for GnRH in primary cultures of sheep pituitaries *(3–5)*. Estradiol treatment of ovariectomized ewes after hypothalamic–pituitary disconnection resulted in an increase in both GnRH receptors *(6–8)* and steady-state amounts of GnRH receptor mRNA *(8)*. Recently, Turzillo et al. *(9)* reported that treatment of ovariectomized ewes with a single injection of estradiol (25 μg) increased relative amounts of mRNA for GnRH receptors and numbers of GnRH receptors within 12 h.

The ability of GnRH to upregulate expression of its own receptor has been well described. Clarke et al. *(10)* observed an increase in amounts of GnRH in hypophyseal blood during the follicular phase of the estrous cycle, indicating that an increase in GnRH secretion may be important in the induction of the preovulatory LH surge. Treatment of ovariectomized ewes with a desensitizing dose of GnRH led to a decrease in the number of GnRH receptors 12 and 24 h after infusion, demonstrating that GnRH is involved in the regulation of its own receptors *(11)*. In ovariectomized ewes after removal of hypothalamic input to the pituitary gland by hypothalamic–pituitary disconnection, the number of GnRH receptors decreased *(12,13)*. When pulses of GnRH were administered to ovariectomized ewes after hypothalamic–pituitary disconnection, the number of receptors for GnRH was restored to similar values as observed prior to hypothalamic–pituitary disconnection *(12,13)*. Thus, it was concluded that GnRH is necessary to maintain the normal number of receptors for GnRH.

The interaction of GnRH and estradiol has also been investigated in ovariectomized ewes after hypothalamic–pituitary disconnection. In ovariectomized ewes after hypothalamic–pituitary disconnection and replacement of pulsatile GnRH, the numbers of GnRH receptors increased 6 h after injection of with estradiol (50 μg). There was a further increase in GnRH receptor numbers after 16 and 20 h of estradiol treatment *(6)*. Finally, in ovariectomized ewes after hypothalamic–pituitary disconnection, estradiol (25 μg), in the absence of GnRH, increased the number of GnRH receptors *(14)*. Thus, the actions of estradiol to increase GnRH receptors prior to the preovulatory LH surge occur independently of GnRH secretion.

The studies described above utilized high levels of estradiol common to the preovulatory period in the ewe. The interactions between GnRH and lower concentrations of estradiol to regulate expression of GnRH receptors during the early follicular phase are not yet known. We hypothesized that basal concentrations of estradiol would upregulate GnRH receptor expression by direct actions at the pituitary regardless of the presence of GnRH. We designed an experiment to determine if there is an interaction between low levels of estradiol and GnRH in long-term ovariectomized ewes after hypothalamic–pituitary disconnection.

Table 1
Amplitude of LH Pulses and Mean Concentrations of LH in Ovariectomized (OVX) Ewes and Ovariectomized Ewes After Hypothalamic–Pituitary Disconnection (HPD)[a]

Treatment	Pulse amplitude, ng/mL	Mean, ng/mL
OVX	0.8^{b}	1.0^{b}
OVX + HPD		
Estradiol/saline	0^{c}	0.2^{c}
Estradiol/GnRH	$0.4^{d,e}$	$0.4^{c,d}$
Placebo/saline	0.008^{c}	0.2^{c}
Placebo/GnRH	$0.6^{b,e}$	0.7^{d}

[a]After hypothalamic–pituitary disconnection, ewes received either estradiol or placebo implants and saline or GnRH (1 pulse/2 h).
[b,c,d,e]Values with different superscripts differ, $p < 0.05$.

Results

Mean LH was greatest in ovariectomized control ewes compared to all other treatments (Table 1; $p < 0.05$). Mean LH and LH pulse amplitude were similar between both GnRH-treated groups (Fig. 1; Table 1). Mean LH and LH pulse amplitude were significantly decreased in all saline-treated animals compared to ovariectomized controls (Table 1; $p < 0.05$). Treatment with an estradiol implant and GnRH significantly increased the amount of GnRH receptor mRNA and number of GnRH receptors compared to ovariectomized controls (Fig. 2; $p < 0.05$). There were no differences in GnRH receptor mRNA or GnRH receptor number between ovariectomized controls and all other treatments (Fig. 2; $p > 0.05$).

Discussion

The results of this experiment suggest that low levels of estradiol in combination with GnRH are required to increase expression of GnRH receptors in ovariectomized ewes after hypothalamic–pituitary disconnection. In this experiment, estradiol alone did not increase expression of GnRH receptors compared to animals with placebo implants or ovariectomized controls. In studies conducted both in vivo *(3,5,7)* and in vitro *(4)*, the number of GnRH receptors increased following administration of estradiol. Likewise, Turzillo et al. *(8)* observed that treatment of ovariectomized ewes after hypothalamic–pituitary disconnection with estradiol (4 implants; 20.9 ± 2.6 pg/mL) in the absence of GnRH input increased expression of GnRH receptors compared to nonsteroid-treated ewes after hypothalamic–pituitary disconnection. The discrepancy in expression of GnRH receptors between this experiment and others probably resides in the doses of estradiol that were administered. In the current experiment, a single estradiol implant

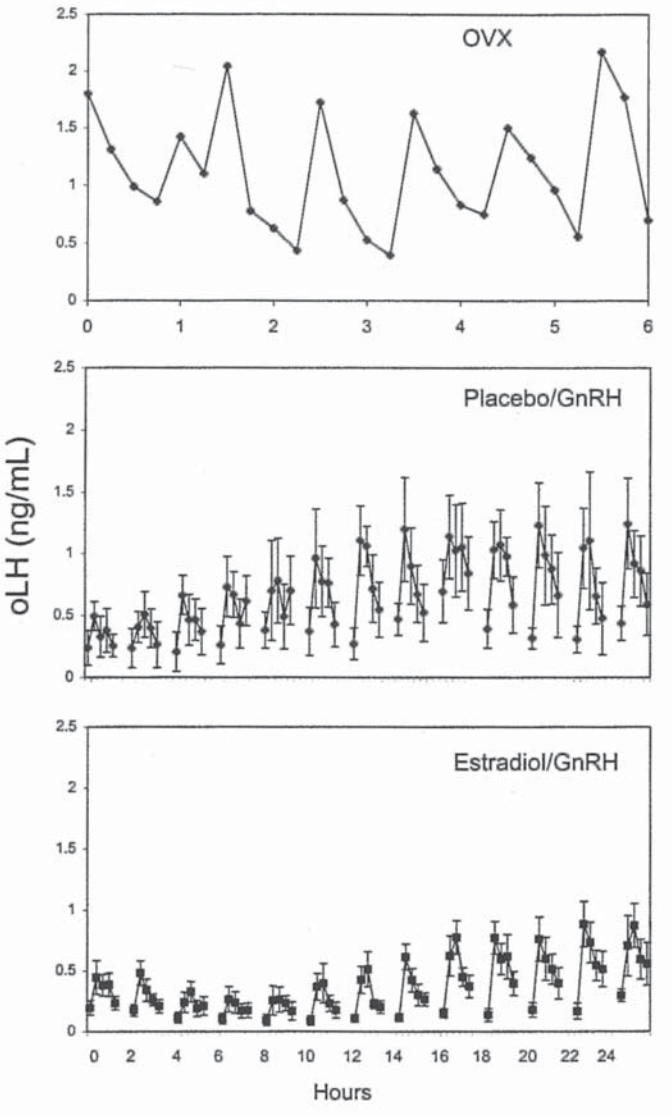

Fig. 1. Serum LH concentrations in a representative ovariectomized control ewe (OVX; upper panel) and mean serum LH in ovariectomized ewes after hypothalamic–pituitary disconnection (lower panels). After hypothalamic–pituitary disconnection, ewes received either estradiol or placebo implants and GnRH at a rate of 100 ng/pulse/2 h.

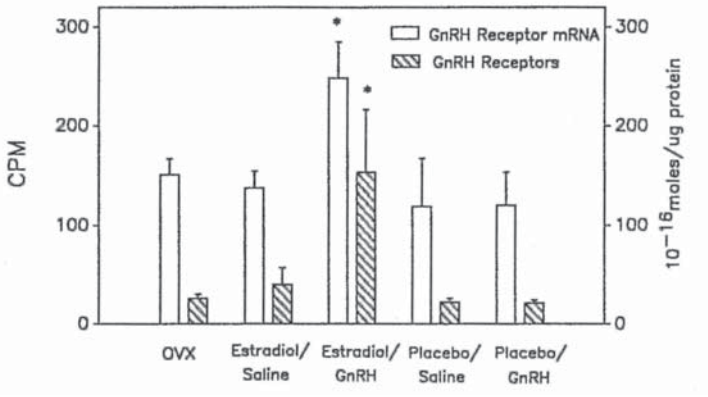

Fig. 2. GnRH receptor expression in ovariectomized control ewes (OVX) and ovariectomized ewes after hypothalamic–pituitary disconnection. After hypothalamic–pituitary disconnection, ewes received either estradiol or placebo implants and saline or GnRH (100 ng/pulse/2 h). Treatment differences denoted by $*p < 0.05$. Data are expressed as mean ± SEM.

was utilized to mimic estradiol levels that are observed during the transition from the late luteal to the early follicular phase of the estrous cycle *(15,16)*. In the in vivo studies cited above, much higher doses (25–50 μg) of estradiol were given as bolus injections or multiple estradiol implants (4 implants) to achieve upregulation of GnRH receptors. In pituitary cultures, lower doses of estradiol (0.004 and 0.04 n*M*) failed to increase the number of GnRH receptors compared to higher doses of estradiol (4.0 and 40.0 n*M*) *(4)*, which were effective in increasing GnRH receptor numbers. Thus, the low level of estradiol in serum during the early follicular phase is apparently, by itself, insufficient to increase the expression of GnRH receptors in the absence of hypothalamic GnRH secretion. It appears that GnRH and estradiol, in the absence of progesterone, interact to upregulate GnRH receptor expression in preparation for the preovulatory LH surge.

The model utilizing ovariectomized ewes after hypothalamic–pituitary disconnection makes a powerful tool to investigate each hormonal input to the pituitary singularly as well as collectively. Using this model, we were able to determine if GnRH, in the presence and absence of low levels of estradiol, was able to increase expression of GnRH receptors. Our results in this study are in agreement with those of Clarke et al. *(6)* in which administration of GnRH in the absence of estradiol to does not increase GnRH receptor numbers. Clarke et al. *(6)* observed that variation in GnRH pulse frequency (1 pulse/h vs 1 pulse/3 h) in the absence of ovarian steroids did not affect the number of GnRH receptors. Similarly, we found that in the absence of estradiol, administration of GnRH pulses at a frequency where pituitary responsiveness was not different from that of OVX controls (Fig. 1; Table 1; placebo/GnRH) did not alter expression of GnRH receptors. Only when the combination of low levels of estradiol and GnRH pulses were administered did we observe an increase in the expression of GnRH receptors in ovariectomized ewes after hypothalamic–pituitary disconnection.

In summary, a single estradiol implant in the absence of GnRH inputs did not change the expression of GnRH receptors compared to ovariectomized controls. However, treatment with low levels of estradiol and GnRH treatment together were sufficient to increase the expression of GnRH receptors compared to all other treatment groups. Based on the lack of effect of GnRH or estradiol alone to upregulate GnRH receptor expression, we conclude that there is an interaction between estradiol and GnRH to increase GnRH receptors and GnRH receptor mRNA in ovariectomized ewes after hypothalamic–pituitary disconnection. Therefore, it is likely that the increase in GnRH receptor expres-

sion that is observed during the early periovulatory period following removal of progesterone is owing to an interaction between estradiol and GnRH.

Materials and Methods

Animals

All studies with animals were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Mature ewes of mixed breeds common to the western United States that had been ovariectomized for at least 2 wk were utilized in this study. The hypothalamic–pituitary interface was surgically disconnected *(15–17)*. One day after hypothalamic–pituitary disconnection, all ewes were fitted with jugular cannulas, and pulses of GnRH (Sigma, St. Louis, MO) were administered (250 ng/4 mL/2-min pulse) at a frequency of 1 pulse/2 h via peristaltic pumps to maintain hypophyseal integrity *(13)*. On the sixth day after hypothalamic–pituitary disconnection, ewes were randomly assigned in a 2×2 factorial arrangement of treatments as follows: half of the ewes received one estradiol-17β (Sigma) containing silastic implant (Dow Corning, Midland, MI) to maintain serum estradiol concentrations comparable to those attained during the luteal phase of the estrous cycle (<5 pg/mL; *15,16*). The remaining ewes received an empty silastic implant (placebo). Ewes with estradiol or placebo implants were then assigned at random to one of the following treatments: (1) saline pulse (estradiol/saline, $n = 5$; or placebo/saline, $n = 5$; 1 pulse/h) or (2) GnRH pulse (estradiol/GnRH, $n = 5$; or placebo/GnRH, $n = 4$; 100 ng pulse/2 h). An additional group of ovariectomized ewes that did not undergo hypothalamic–pituitary disconnection served as controls (OVX; $n = 4$). Following insertion of implants, GnRH and saline pulses were administered as rapid iv boluses. Blood samples were collected 15 min prior to each pulse of GnRH or saline and at 15-min intervals for 1 h after each pulse until tissues were collected. Twenty-four hours after implant insertion, anterior pituitary glands were collected, cut along the sagittal plane, and immediately frozen at –70°C. Hypothalamic–pituitary disconnection was verified at the time of tissue collection by the presence of an aluminum barrier that was inserted at the time of surgery and later by serum LH concentrations. Animals in which hypothalamic–pituitary disconnection was not complete were omitted from analysis.

mRNA Analysis

Total cellular RNA was isolated from half of each pituitary using a modification of the one-step method *(18)* and Trizol (1 mL/100 mg tissue; Gibco BRL, Grand Island, NY). Polyadenylated RNA was isolated from total cellular RNA using oligo dT cellulose chromatography (Qiagen, Santa Clarita, CA). Integrity of mRNA was determined by Northern blot analysis (data not shown). Changes in steady-state amounts of GnRH receptor mRNA were quantitated by slot-blot analysis *(9,19)*. One microgram of poly(A^+) mRNA was denatured in 100 m*M* NaOH, 1 m*M* EDTA, applied to a nylon filter (Zeta Probe; Bio-Rad, Hercules, CA) on slot blots, and crosslinked to the nylon filter by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). All animals were analyzed on the same slot blot. Using a random-primed ^{32}P-labeled ovine GnRH receptor cDNA probe *(9)* blots were hybridized overnight at 45°C and washed in a final solution of 0.1X SSC + 0 .1% SDS at room temperature. Bands of ^{32}P-cDNA:mRNA heteroduplexes were visualized with autoradiography and cpm bound quantitated (Instant Imager; Packard, Meridan, CT). Blots were stripped of radioactivity by boiling for 30 min in 0.1X SSC + 0.1% SDS and rehybridized with a cDNA probe encoding rat cyclophilin *(20)* to correct for equal loading of mRNA in each slot. Results are expressed as relative amounts of GnRH receptor mRNA per unit of cyclophilin mRNA (cpm GnRH receptor mRNA/cpm cyclophilin mRNA).

Radioimmunoassays

Serum concentrations of LH were determined by radioimmunoassay *(21)*. The reference preparation for oLH was NIADDK-ovine LH-1-3 as tracer and NIADDK-ovine LH-25 as standard. The sensitivity of the assay was 0.15 ng/mL; intra-assay and interassay coefficients of variation were 12.5 and 11.3%, respectively.

GnRH Receptor Assays

Numbers of GnRH receptors were determined in a single radioreceptor assay using a partially purified membrane fraction prepared from ovine pituitaries as a standard curve *(11)*. D-Ala6-Des-Gly10-GnRH-ethylamide was obtained from Sigma. Protein was quantitated on half of each pituitary (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA) *(22)*.

Statistical Analysis

Data were analyzed by ANOVA and Duncan's New Multiple-Range Test *(23)* with the SPSS statistical package (SPSS, Inc., Chicago, IL). Pulses of LH in OVX ewes were identified using the Cluster Analysis Program *(24)*. LH pulse amplitude was determined by subtracting the nadir from the peak height. Data were expressed as mean ± SEM. Values of $p \leq 0.05$ were considered to be significant.

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