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Estradiol Up-Regulation of Pituitary Progesterone Binding Is Required for Progesterone Inhibition of Luteinizing Hormone Release

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This study examines the regulation of progesterone receptor (PR) in the inhibition of pituitary luteinizing hormone (LH) secretion. Ovariectomized ewes underwent hypothalamic-pituitary disconnection, were pulsed with gonadotropin-releasing hormone (GnRH) and received 1 of 4 treatments: estradiol alone (E), estradiol priming before progesterone (E+P), E removed and replaced with P (E-P), or no steroids (C). P treatment for 24 h, with E or following E-priming, reduced LH pulse amplitude by 55% ($p < 0.05$). E alone did not affect LH release. E increased pituitary cytosolic P binding capacity fourfold over controls ($p < 0.01$) and P further increased binding to eight times controls ($p < 0.01$). Pituitary PR mRNA increased to 149 and 171% of C in E and E+P groups, respectively ($p < 0.05$), but E removal resulted in PR mRNA levels not different from controls. Pituitary receptors for GnRH were tripled by E alone compared to C ($p < 0.01$), whereas P alone or with E had no effect. These data suggest an E-induced, direct pituitary inhibition of LH secretion by P and that this effect of P is associated with E-enhanced binding of P in the pituitary. Additionally, the direct pituitary effects of P on LH secretion cannot be accounted for by influences on GnRH receptor numbers.

Key Words: Progesterone receptor; GnRH receptor; hypothalamic-pituitary disconnection; sheep; multiplex PCR.

Introduction

Tonic luteinizing hormone (LH) release during the luteal phase of the estrous cycle of the ewe is influenced by estradiol and progesterone. Recent evidence suggests that ovarian steroids exert their effects both directly on the pituitary and indirectly via hypothalamic regulation of LH secretion (Girmus and Wise, 1992). At the hypothalamic level, estradiol reduces LH secretion by increasing the sensitivity of the GnRH pulse generator to progesterone negative feedback (Karsch et al., 1987), possibly by up-regulating progesterone receptors in the hypothalamus (Lauber et al., 1991; Bayliss et al., 1991). Additionally, estradiol appears to reduce LH stores in the pituitary by altering GnRH-stimulated LH synthesis (Nett et al., 1990; Girmus and Wise, 1992).

Direct inhibition of pituitary LH secretion in vivo by progesterone and estradiol occurs when steroids act in a synergistic fashion (Clarke et al., 1989; Girmus and Wise, 1991). Neither steroid alone prevents LH secretion by direct action on the pituitary, but if the pituitary is primed with estradiol, LH secretion is effectively inhibited by progesterone (Batra and Miller, 1985, 1986; Krey and Kamel, 1990; Krey et al., 1990; Girmus and Wise, 1992). These studies suggest that, in order to achieve effective progesterone inhibition of pituitary activity, estradiol may be required to increase the number of progesterone receptors in the pituitary. Furthermore, progesterone and estradiol receptors are present in gonadotrophs (Sprangers et al., 1989; Fox et al., 1990) and estradiol induction of pituitary progesterone receptor concentrations in vitro is associated with the ability of progesterone to decrease LH secretion (Krey and Kamel, 1990).

The study reported herein examined the regulation of progesterone binding in the pituitary and changes in steady-state levels of progesterone receptor mRNA associated with ovarian steroid-mediated inhibition of LH secretion. Second, in this study we attempted to determine if steroid inhibition of LH release is associated with altered GnRH receptor numbers in the pituitary. Hypothalamic-pituitary disconnected ewes infused with a constant frequency of GnRH pulses were used to examine steroid action on the pituitary and distinguish the pituitary versus hypothalamic effects of ovarian steroids.

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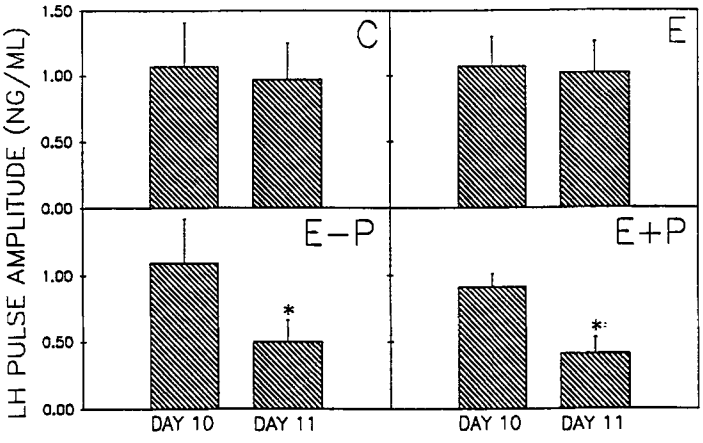

Fig. 1. Mean (±SEM) values for LH pulse amplitude (pulse height minus the preceding nadir) from HPD-OVX ewes pulsed with exogenous GnRH with and without steroid treatment. Treatment groups: no steroids (C; $n = 5$); estradiol implant on d 6 (E; $n = 4$); estradiol implant on d 6, removed on d 10 and replaced with progesterone implant (E-P; $n = 4$); estradiol implant on d 6, progesterone added on d 10 (E+P; $n = 4$). P reduced mean LH pulse amplitude by 55% in both groups treated with E ($p < 0.05$).

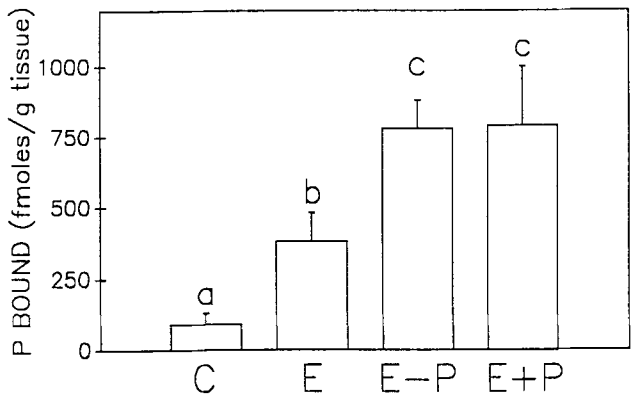

Fig. 2. Pituitary cytosolic progesterone binding capacity in HPD-OVX ewes infused with exogenous GnRH and treated with steroids. Mean (±SEM) values for total pituitary cytosolic progesterone binding capacity were determined in the four groups of ewes described in the legend of Fig. 1. E raised P binding capacity fourfold ($p < 0.05$) and P raised P binding capacity eightfold in E-treated ewes ($p < 0.05$) compared to controls.

Results

LH secretion was measured in hypothalamic-pituitary disconnected (HPD) ovariectomized ewes receiving pulsatile GnRH. Ewes treated with estradiol (E) or blank implants (C) were tested 4 d after implantation (d 10) and after 1 d of progesterone treatment (d 11) (Fig. 1). No significant differences in LH pulse amplitude (pulse height minus the preceding nadir) were observed between samples collected on d 10 and 11 in controls or E-treated ewes. In contrast, P treatment for 1 d, either combined with E (E+P) or following E removal (E-P), decreased LH pulse amplitude by 55% ($p < 0.05$) (Fig. 1). Cytosolic P binding capacity was determined in pituitary tissues collected after the final blood sampling on d 11. P binding capacity was raised by E treatment fourfold compared to controls ($p < 0.01$) and eightfold compared to controls ($p < 0.01$) when P was combined with E priming or treatment (groups E-P and E+P, respectively) (Fig. 2). The nuclear fraction of these pituitary preparations was also assayed for P binding capacity, but values were too low to reliably estimate specific binding.

The effect of steroid treatment on pituitary PR mRNA levels was examined by comparing ratios of PCR-amplified PR cDNA to GAP-D cDNA transcribed from mRNA isolated from pituitary tissues collected from ewes in various treatment groups on d 11. Normalization of PR cDNA to GAP-D cDNA adjusted the PR cDNA values for differences between samples in RNA quality or pipetted volumes. GAP-D cDNA was not affected by the treatments used in this study; mean log cDNA values for GAP-D for each treatment group coamplified in a single reaction tube with PR primers were 1.76 ± 0.06 (C), 1.87 ± 0.11 (E), 1.76 ± 0.23 (E-P), and 1.75 ± 0.61 (E+P). Treatment of ovariectomized, hypothalamic-pituitary disconnected ewes with E alone increased PR cDNA/GAP-D cDNA in the pituitary to 149% of control levels ($p < 0.05$). In comparison, PR/GAP-D ratios in ewes 24 h after E removal and replacement with P were not different from controls (0.67 ± 0.5 for E-P versus 0.54 ± 0.11 for C; Fig. 3).

Estradiol implants increased pituitary GnRH receptors nearly threefold over levels in controls ($p < 0.01$; Fig. 4). One day (24 h) of P treatment (groups E-P and E+P) resulted in GnRH receptor concentrations not significantly different from controls.

Discussion

Work done previously in this and other laboratories that suggests the inhibition of GnRH-stimulated LH release by progesterone requires estradiol (Karsch et al., 1977; Wheaton and Mullet, 1982; Batra and Miller, 1985, 1986; Clarke et al., 1989; Krey and Kamel, 1990; Krey et al., 1990; Girmus and Wise, 1991, 1992). In the current study, we observed an eightfold increase compared to controls in the binding of progesterone to pituitary cytosolic progesterone receptors following treatment with estradiol and progesterone. Concurrent with the increase in pituitary progesterone binding, we recorded dramatic reductions in LH release. Although progesterone binding capacity increased to a lesser extent following estradiol treatment alone, no significant changes were observed in LH release in this treatment group. These findings further support the hypothesis that estradiol readies the pituitary for the inhibitory effects of progesterone but does not contribute in a major way to the negative regulation of LH release during the luteal phase in the ewe. We suggest that, with estradiol priming, sufficient progesterone receptor is induced and present on pituitary gonadotrophs for the inhibitory action

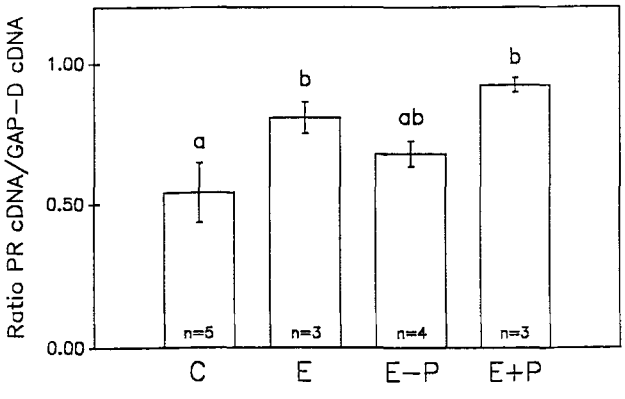

Fig. 3. Progesterone receptor (PR) cDNA coamplified together with GAP-D cDNA from pituitary mRNA by multiplex polymerase chain reaction (MPCR). Treatment groups are described in legend for Fig. 1. Mean ratios (± SEM) of log values for PR to GAP-D cDNA were increased in E and E+P treatment groups to 149 and 171% of controls, respectively ($p < 0.05$), but removal of E implants and replacement with P resulted in PR/GAP-D ratios not different from controls. (RNA samples for one animal in each E and E+P treatment groups were lost in preparation; $n = 3$ for these groups.)

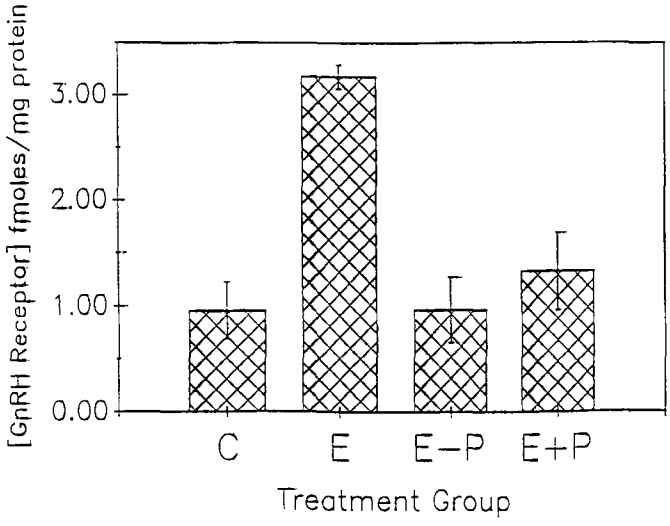

Fig. 4. Mean (±SEM) concentrations of GnRH receptors in pituitaries collected after blood sampling on d 11 of steroid treatment (*see* Fig. 1 legend for description of treatment groups). GnRH receptor concentrations were determined by a standard curve method. E raised GnRH receptor concentrations more than threefold above control levels ($p < 0.01$). A single day of P reversed the GnRH receptor increase.

of progesterone to be apparent. Progesterone alone is reportedly incapable of suppressing LH release in GnRH-treated HPD ewes (Clarke et al., 1989; Girmus and Wise, 1991) and the negligible progesterone binding in ovariectomized ewes is likely owing to insufficient numbers of progesterone receptors in the absence of estradiol. Conversely, the presence of progesterone in the pituitary preparations of those animals treated with progesterone may have contributed to the higher levels of progesterone binding in those animals.

This report of the enhancement of progesterone binding in the pituitary is unique in that it examines the role of steroid hormones on pituitary LH release independent of hypothalamic influence. There are confirming reports of up-regulation of progesterone receptors in certain uterine tissues (Lessey et al., 1988; Clarke, 1991). The mixed cell population present in the pituitary and used in our binding assays may include cells whose progesterone receptors are affected by steroid treatment but do not contribute to the regulation of LH release. Other studies have observed specific increases in progesterone receptors in gonadotrophs following estradiol treatment (Sprangers et al., 1990). Estradiol treatment increased PCR-amplifiable pituitary PR mRNA after 4 d of treatment and this suggests that steady-state levels of progesterone receptor mRNA was increased by estradiol. It follows that, as progesterone binding in the pituitary increases so dramatically following estradiol treatment, the synthetic machinery for the production of progesterone receptor might also be increased.

Although progesterone further increased its own binding in the pituitary, it did not significantly increase PR mRNA levels above those induced by E alone. It was not clear if the slightly lower PR mRNA levels observed in the E-P group were owing to the removal of E implants or 24 h of P treatment. It is possible that mechanisms regulating the synthesis and expression of PR in the pituitary are not the same mechanisms regulating PR transcription. There is a recent report of possible differential regulation of PR mRNA and PR protein in the primate corpus luteum (Duffy and Stouffer, 1995). Additionally, progesterone has been shown to shorten the length of poly-A tails in LH-beta mRNA (Wu and Miller, 1991), although it does not directly alter its transcription (Hamernik et al., 1987; Girmus and Wise, 1991; Wu and Miller, 1991). Reduction in poly-A tail length may render the mRNA molecule less efficiently translated (Jackson and Standart, 1990) or more susceptible to degradation (Wilson et al., 1978). Perhaps this negative action of progesterone affects PR mRNA as well. If this is true in the present case, increasing the length of progesterone treatment should lead eventually to reduced progesterone binding in the pituitary. Indeed, pituitary progesterone receptors were homologously down-regulated in estradiol-treated monkeys implanted simultaneously with progesterone for 14 d (Sprangers et al., 1990). Nonetheless, an eventual recovery from progesterone-induced LH inhibition by reduced progesterone binding might not be expected because the negative influence of progesterone on mRNA exists for LH-β mRNA as well.

In this study, treatment with estradiol alone did not affect pituitary LH secretion. The increase in GnRH receptor numbers by estradiol treatment may have led to the slight rise in LH release as induction of GnRH receptors in vitro is clearly dose-dependent (Gregg et al., 1990). The

effect of the removal of estradiol implants on LH pulse amplitude was not rigorously examined in the current study. Had the removal of estradiol significantly reduced LH release, however, the serum LH profiles, pituitary progesterone binding capacity, PR mRNA, and GnRH receptor concentration results of the two groups treated with progesterone following the removal of estradiol implants or combined with estradiol treatment might have been less similar.

The frequency of GnRH pulses chosen for the present study (one pulse every 2 h) reflects the frequency of LH pulses observed during the luteal phase in the ewe (Karsch et al., 1980). If differences in GnRH pulse frequency affect steroid action on the pituitary, our studies using only the one pulse frequency may not accurately address the role of the hypothalamus in steroid-induced LH suppression. Although we have attempted to mimic the number of LH pulses observed per hour during the luteal phase, using LH release as an indicator of GnRH release may not be ideal.

The mechanism whereby progesterone directly inhibits pituitary LH secretion is unknown. Progesterone reduces both GnRH receptor concentrations (Laws et al., 1990) and levels of GnRH receptor mRNA (Sealfon et al., 1990). These observations are associated with reduced LH secretion in these studies. We observed similar results in ewes treated with progesterone after estradiol priming. Although estradiol alone dramatically increased GnRH receptor concentrations (more than three times control levels), LH pulse amplitude was only slightly increased above controls. In contrast, treatment with progesterone for 1 d reduced GnRH receptor concentrations to controls levels. In addition to events affecting GnRH receptor concentrations, other mechanisms may be involved in the regulation of LH release (Wise et al., 1984). Progesterone effects on LH secretion appear to require ribonucleic acid and protein synthesis (Krey et al., 1990; Turgeon and Waring, 1991) and could involve regulation of G-protein concentrations (Davidson et al., 1991; Bouvier et al., 1991). Recent observations suggesting that GnRH self-priming requires the progesterone receptor (Waring and Turgeon, 1992) and observations of progesterone receptor-mediated transcription by phosphorylation (Denner et al., 1990; Power et al., 1991) indicate that regulation of this receptor may play a major role in regulating gonadatroph function.

The present study offers important information regarding the role of gonadal steroids in the regulation of LH release in an animal model where pituitary function can be scrutinized independently of hypothalamic influence. Other investigators using hypothalamic-pituitary intact animals have described estradiol- dependent inhibition of hypothalamic GnRH secretion by progesterone (Karsch et al., 1980, 1987). Thus, estradiol-induced progesterone inhibition of LH secretion during the luteal phase of the estrous cycle in the ewe likely occurs via direct regulation of pituitary function combined with regulatory actions in the hypothalamus.

In summary, progesterone inhibits pituitary LH release in estradiol-primed ewes. The fall in LH is associated with an estradiol-dependent increase in progesterone binding in the pituitary. Although progesterone receptor mRNA and concentrations of GnRH receptor in the pituitary are also increased by estradiol treatment, these parameters were not associated with decreased LH release.

Materials and Methods

Animals and Treatments

The hypothalamic-pituitary interface was surgically disconnected (HPD) in seventeen ovariectomized (OVX) western range ewes (Clarke et al., 1983). Three days later, jugular infusions of 400 ng GnRH in 4 mL sterile saline delivered over 2 min every 2 h were initiated and continued throughout the experiment (Girmus and Wise, 1991). Ewes were divided randomly into four groups. One group received no steroid treatment (C; $n = 5$). The other three groups were administered estradiol via subcutaneous silastic implants after 6 d of GnRH infusion (d 6). On d 10, estradiol implants were removed in one group and replaced with progesterone implants for 1 d (E-P; $n = 4$). Another group received progesterone implants on d 10 but the estradiol implants were not removed (E+P; $n = 4$). In the third group, estradiol implants remained in place through d 11 (E; $n = 4$). Steroid implants were constructed as described previously and one 30–mm estradiol implant was used to raise serum estradiol concentrations approx 3–4 pg/mL and serum progesterone concentrations approx 2 ng/mL over preimplantation values (Girmus and Wise, 1991).

Sample Collection and Assays

Serum LH

To measure LH release in response to exogenous GnRH, blood samples were collected at –20, –10, 0, 10, 20, 30, 40, 50, and 60 min relative to an exogenous GnRH pulse. Blood samples were collected around exogenous GnRH pulses on d 10 before progesterone implantation and 1 d later (d 11). Serum was harvested and stored at –20°C until assayed for LH by RIA (Niswender et al., 1969; Girmus and Wise, 1991).

Values for LH pulse amplitude (pulse height minus the preceding nadir) in individual ewes were determined and mean values ± SEM for pulse amplitude on different days for each treatment were calculated.

Progesterone and GnRH Receptor-Binding Assays

After the last blood sample was taken on d 11, ewes were given an overdose of pentobarbital and exsanguinated. Surgical separation of the pituitary from the hypothalamus was verified by visual examination. Pituitaries were removed, trimmed free of connective tissue, and halved. One half was snap-frozen in liquid nitrogen for later RNA isolation and the other half was minced and suspended immediately in ice-cold TG buffer (10 m*M* Tris,

30% glycerol v/v, pH 7.4) for subsequent analysis (within 1 h) of total protein concentration and progesterone binding capacity.

Progesterone binding capacity was determined in pituitary tissues using ^{3}H-progesterone as ligand in a receptor-binding assay (Clarke et al., 1987). In initial experiments, Scatchard analysis of pituitary cytosol preparations from intact and estradiol-treated ewes was performed. Saturation of ^{3}H-progesterone binding occurred at approx 8 n*M* and Kd of 2.3 n*M* was observed. For subsequent saturation analysis, [1,2,6,7-^{3}H]Progesterone (Amersham, Arlington Heights, IL) was used as the labeled ligand. Pituitary halves were weighed, minced, and suspended in ice-cold TG buffer with tissue/volume = 40 mg/mL. Suspensions were homogenized with a Brinkman homogenizer (Brinkman Instruments, Westbury, NY) and centrifuged at 35,000*g* for 1 h. The cytosolic suspension was added to tubes containing 12 or 15 n*M* ^{3}H-Progesterone with or without 100–fold excess of cold progesterone in a final volume of 0.5 mL and incubated at 30°C for 45 min. Following incubation, 0.25 mL of ice-cold DCC solution (1.0% charcoal, 0.95% dextran in TG buffer) was added to the tubes with vortexing and tubes were immediately centrifuged at 2500*g* for 6 min. The supernatant was then decanted and counted in a scintillation counter.

Pituitary plasma membranes were recovered following removal of the cytosolic suspension used in progesterone binding assays. Membranes were reconstituted and GnRH receptor numbers determined using a standard curve technique (Nett et al., 1981).

Progesterone Receptor Message RNA Quantitation

Total pituitary RNA was isolated using a guanidine isothiocyanate/cesium chloride separation technique (Chirgwin et al., 1979; Girmus and Wise, 1991). RNA was quantified spectrophotometrically and 1 μg total RNA was converted to cDNA by reverse transcription (AMV, Promega, Madison, WI). A multiplex polymerase chain reaction (MPCR) assay (Chamberlain et al., 1988) was developed in which a 270–bp fragment of the progesterone receptor message was coamplified in a single reaction vial with a 318-bp fragment of the message for glyceraldehyde 3-phosphate dehydrogenase (GAP-D), a constitutively expressed protein (Tso et al., 1985). Human primers for progesterone receptor (PR; 0.4 μ*M* each primer; Sequences: GGGGATGAAGCATCAGGCTGT, CTGT GGGAGAGCAACAGCATC) and GAP-D (0.2 μ*M* each primer; Sequences: TGGAGAAGGCTGGGGCTC, GC [AT]GGGATGATGTTCTGG) were added together to reverse-transcribed DNA in a reaction containing 3.125 m*M* $MgCl_2$, 250 μ*M* each dNTP, and 5 U Taq-polymerase (Promega) in 1X PCR buffer. Primers were chosen for their specificity and high homology between human, rodent, and avian species. The progesterone receptor PCR product spans most of the highly conserved DNA-binding C-region of the progesterone receptor cDNA similar in both A and B forms of the receptor (Misrahi et al., 1987; Kastner et al., 1990). In initial experiments, primer concentrations, temperatures, and cycle number in the multiplex reaction were optimized. Twenty-eight cycles produced amplification products from each set of primers in the linear range for the reaction conditions used.

Quantitation of progesterone receptor cDNA was accomplished by first separating the amplified PR cDNA and GAP-D cDNA products by electrophoresis through 10% polyacrylamide gel (19:1 acrylamide:BIS acrylamide). Bands of cDNA were then visualized using a silver stain (Bio-Rad, Hercules, CA), and scanned using an ImageQuant scanning densitometer (Molecular Dynamics, Sunnyvale, CA). Optical density values for each band were recorded, and PCR-produced PR cDNA values were normalized to GAP-D cDNA values from the same reaction tube and ratios were compared between samples from ewes in each treatment group. RNA samples for one animal in each E and E+P treatment groups were lost in preparation; $n = 3$ for these groups.

Statistics

Valid pulses of LH were determined using the computer algorhythm program, Cluster (Veldhuis and Johnson, 1986). Differences in values from ewes in each treatment group were tested for significance by one-way analysis of variances (ANOVAs) for each parameter examined: LH pulse amplitude, total cytosolic pituitary P binding capacity, PCR-amplifiable PR mRNA (expressed as ratios of PR cDNA/GAP-D cDNA), and GnRH receptor concentration. Least significant difference tests were performed on data sets with significant differences by ANOVA.

Acknowledgments

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