INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313-761-4700 800-521-0600
Direct ovarian steroid regulation of pituitary luteinizing hormone secretion, stores and subunit mRNA

Girmus, Ronald Leslie, Ph.D.
The University of Arizona, 1992
DIRECT OVARIAN STEROID REGULATION OF PITUITARY
LUTEINIZING HORMONE SECRETION, STORES AND SUBUNIT mRNA

by
Ronald Leslie Girmus

A Dissertation Submitted to the Faculty of the
COMMITTEE ON ANIMAL PHYSIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1992
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Ronald Leslie Girmus entitled DIRECT OVARIAN STEROID REGULATION OF PITUITARY LUTEINIZING HORMONE SECRETION, STORES AND SUBUNIT mRNA and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Mark E. Wise

Ronald E. Allen

Barry S. Komm

Vincent Guerriero

Patricia B. Hoyer

Date

4-16-92

4-16-92

4-16-92

4-16-92

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Mark E. Wise

Date

4-16-92
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Ronald L. Price
TABLE OF CONTENTS

List of Figures..........................................................5

Abstract.................................................................7

Chapter 1. Literature review..........................................8

Chapter 2. Introduction...............................................13

Chapter 3. Direct pituitary effects of estradiol and progesterone on luteinizing hormone release, stores and subunit messenger ribonucleic acids.
   Abstract...........................................................15
   Introduction.........................................................16
   Methods and Materials..............................................19
   Results...............................................................22
   Discussion..........................................................24

Chapter 4. Progesterone directly inhibits pituitary LH secretion in an estradiol-dependent manner.
   Abstract...........................................................43
   Introduction.........................................................44
   Methods and Materials..............................................45
   Results...............................................................47
   Discussion..........................................................48

Chapter 5. Progesterone binding associated with direct, estradiol-dependent inhibition of LH secretion by progesterone.
   Abstract...........................................................60
   Introduction.........................................................61
   Methods and Materials..............................................62
   Results...............................................................65
   Discussion..........................................................65

Chapter 6. Summary.....................................................73

Appendix 1. Methods and Materials..................................75

References....................................................................98
LIST OF FIGURES

Figure 1. Mean LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and after steroid treatment. ....32

Figure 2. Mean serum LH profiles (±SEM) of HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. ............................................... 33

Figure 3. Mean values for LH pulse amplitude, serum LH concentration and baseline LH concentration in HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. ....................... 34

Figure 4: Photographs of autoradiograms confirming identity of mRNA being examined. ..............................35-36

Figure 5: Photographs of autoradiograms of slot blots determining linear range. ............................37-38

Figure 6: Average density of hybridization signals of 0.1 to 1.5 ug of total pituitary RNA probed for LH-β subunit, LH-α subunit or GH mRNA. ..................................................39

Figure 7: Photographs of autoradiograms of slot blots determining relative levels of LH-β subunit, α-subunit and GH mRNA. ..................................................40-41

Figure 8. Relative α-subunit mRNA, LH-β subunit mRNA and LH tissue concentrations in HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. ............42

Figure 9. The mean (±SEM) serum LH profile of HPD-OVX ewes infused with exogenous GnRH before and 12 to 48 hours after exchanging blank implants. ..............................................53

Figure 10. The mean (±SEM) serum LH profile of HPD-OVX ewes infused with exogenous GnRH before and 12 to 96 hours after estradiol implantation. ........................................54

Figure 11. The mean (±SEM) serum LH profile of estradiol-treated HPD-OVX ewes before and 0.5 to 96 hours after estradiol withdrawal. ..................................................55

Figure 12: The mean (±SEM) serum LH profile of estradiol-treated HPD-OVX ewes before and 0.5 to 96 hours after estradiol withdrawal and progesterone implantation. .......56
Figure 13. Mean (±SEM) values for LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and 0.5 to 96 hours after various steroid treatments. .......................... 57

Figure 14. Mean (±SEM) values for LH pulse amplitude were plotted against time and the least-squares-fit curve determined in HPD-OVX ewes infused with exogenous GnRH, primed with estradiol and treated with blank or progesterone implants. ........................................ 58

Figure 15. Mean (±SEM) values for serum progesterone and LH pulse amplitude were determined before and 0.5 to 96 hours after progesterone implantation in estradiol-primed HPD-OVX ewes infused with exogenous GnRH. ............................ 59

Figure 16. Mean (±SEM) values for LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and after steroid treatment. .................................................. 70

Figure 17. Mean (±SEM) serum LH profiles of HPD-OVX ewes infused with exogenous GnRH and treated with steroids. .......... 71

Figure 18. Pituitary cytosolic progesterone receptor binding capacity in HPD-OVX ewes infused with exogenous GnRH and treated with steroids. ............................... 72
ABSTRACT

The ovarian steroids, progesterone and estradiol, regulate luteinizing hormone synthesis and secretion during the estrous cycle of mature ewes. During the luteal phase of the cycle the ovarian steroids inhibit luteinizing hormone secretion. Luteinizing hormone is secreted from the pituitary when stimulated by the hypothalamic neuropeptide, gonadotropin-releasing hormone. Ovarian steroids can inhibit luteinizing hormone secretion indirectly, by decreasing the secretion of gonadotropin-releasing hormone or directly, by modulating the response of the pituitary to gonadotropin-releasing hormone. These studies have examined the direct control of pituitary luteinizing hormone secretion by using an in vivo model in which endogenous gonadotropin-releasing hormone (GnRH) release has been ablated and replaced with exogenous GnRH release at a constant frequency. Progesterone directly inhibited pituitary LH secretion in an estradiol-dependent manner and this may not require inhibition of pituitary LH synthesis. Progesterone inhibition of pituitary luteinizing hormone secretion is associated with enhanced progesterone binding by the pituitary.
The basis for regulation of ovarian functions such as: oogenesis, folliculogenesis, ovulation, corpus luteum formation/activation, and ovarian steroidogenesis is the temporal series of endocrine events that comprise each estrous/menstrual cycle [Freeman ME, 1988]. The estrous/menstrual cycle can be divided into follicular and luteal phases. In the ewe, the follicular phase is characterized by follicular growth, selection and final maturation of a pre-ovulatory follicle, which ovulates, marking the end of the follicular phase. The luteal phase is marked by the formation and regression of the corpus luteum that develops from the ruptured pre-ovulatory follicle. Prominent endocrine events during estrous/menstrual cycles include: a LH surge causing ovulation, the rise and fall of progesterone secretion in the luteal phase, and a follicular phase rise in estradiol triggering the next LH surge and subsequent ovulation. Superimposed upon these events is ongoing oogenesis and folliculogenesis.

In the ewe, the hormonal events occurring in each estrous/menstrual cycle are the result of coordinated endocrine interactions between the hypothalamus, pituitary, ovary and uterus [Goodman RL, 1988]. Secretion of GnRH from the hypothalamus stimulates synthesis of LH and FSH and
stimulates "surge-like" and tonic release of LH and FSH [Clarke and Cummins, 1982; Cummins and Clarke, 1985; Karsch FJ, 1987; Fink G, 1988]. In the rat and ewe, LH and FSH control follicular selection/maturation [Richards JS, 1980; Greenwald and Terranova, 1988], ovulation [Lipner, 1988], ovarian steroidogenesis [Gore-Langton and Armstrong, 1988] and in the ewe, corpus luteum formation [Niswender and Nett, 1988]. In the ewe, corpus luteum regression is controlled through the interaction of pituitary LH and uterine prostaglandins in some species [Goodman RL, 1988]. In the ewe and monkey, ovarian steroids feedback on the hypothalamus and pituitary to regulate GnRH [Clarke IJ, 1987; Karsch et al., 1987; Karsch FJ, 1987; Plant TM, 1986], LH [Clarke and Cummins, 1984; Gharib et al., 1990] and FSH synthesis and secretion [Clarke and Cummins, 1984; Mercer et al., 1989; Findlay and Clarke, 1984; Gharib et al., 1990]. In many species, LH is central to regulation of ovarian events during each estrous/menstrual cycle [Martin GB, 1984]. In the ewe, LH secretion in the follicular phase drives the final maturation of the pre-ovulatory follicle, creating a rise in estradiol that triggers the LH surge. The surge release of LH during the cycle causes ovulation and the tonic release of LH in the luteal phase is required for corpus luteum function [Goodman RL, 1988; Niswender and Nett, 1988]. The fall in progesterone levels
following corpus luteum regression allows LH pulse frequency to increase, repeating the cycle.

The interactions of progesterone and estradiol with the hypothalamic-pituitary axis regulate LH synthesis and secretion during the estrous/menstrual cycle. In the ewe a rise in estradiol in the follicular phase, coupled with permissively low progesterone concentrations stimulates the pulsatile release of LH [Karsch et al., 1983], further increasing estradiol secretion which eventually triggers the LH surge, causing ovulation [Karsch FJ, 1987; Scaramuzzi et al., 1971; Kaynard et al., 1988]. If progesterone concentrations remain high, estradiol cannot trigger the LH surge [Karsch FJ, 1987]. After ovulation, the rise in progesterone acts synergistically with low estradiol concentrations to inhibit tonic release of LH [Karsch et al., 1980; Goodman et al., 1981], allowing normal formation and regression of the corpus luteum in the luteal phase [Auletta and Flint, 1988].

In many species, ovarian steroids can regulate LH synthesis and secretion via the control of GnRH release from the hypothalamus or in the pituitary by modulating GnRH stimulation of LH synthesis and release [Karsch FJ, 1987; Gharib et al., 1989; Mercer JE, 1990]. In the ewe, in the follicular phase of the estrous/menstrual cycle, estradiol, in conjunction with permissively low progesterone concentrations,
increases the frequency of GnRH pulsatile release, thereby increasing the frequency of LH pulsatile release [Karsch et al., 1979; Goodman et al., 1980; Karsch et al., 1983; Kaynard et al., 1988]. In the monkey and ewe, the continuing rise in estradiol increases pituitary sensitivity to GnRH [Knobil E, 1981] and triggers a surge of GnRH secretion [Karsch, 1990]. This combination of endocrine events is responsible for generating the LH surge in the ewe [Karsch FJ, 1987; Goodman RL, 1988]. In the luteal phase of the estrous cycle of the ewe, estradiol and progesterone act synergistically to inhibit GnRH secretion [Goodman et al., 1981; Karsch FJ, 1987; Goodman RL, 1988]. LH subunit mRNA concentrations are decreased, partially due to depressed GnRH secretion [Corbani et al., 1990] and these effects contribute to suppressed LH secretion during the luteal phase. The direct pituitary actions of progesterone and estradiol in the luteal phase remain ambiguous and the integration of hypothalamic and pituitary regulation by steroids in the luteal phase is unknown.

Progesterone may directly inhibit pituitary LH release in an estradiol-dependent manner. Progesterone and estradiol receptors are present in the gonadotrophs of rats and monkeys but not in lactotrophs [Sprangers et al., 1989; Fox et al., 1990] and estradiol enhances progesterone receptor concentrations in gonadotrophs of monkeys [Sprangers et al., 1990]. In vitro studies demonstrate progesterone's estradiol-
CHAPTER 2
INTRODUCTION

The ovarian steroids, progesterone and estradiol, feed back on the hypothalamic-pituitary axis to regulate LH secretion during the estrous cycle of ewes. Regulation of LH secretion may occur indirectly, at the level of the hypothalamus, through control of gonadotropin-releasing hormone (a neuropeptide which stimulates pituitary LH secretion) secretion or directly at the level of the pituitary, through modulation of pituitary response to GnRH.

Both progesterone and estradiol are required for ovarian control of LH secretion. The intrapituitary actions of these hormones may involve the regulation of LH synthesis and storage, thus controlling the concentration of LH available for secretion. Hetero- and homologous regulation of steroid receptors may also be involved in the control of LH secretion.

These studies will distinguish the hypothalamic (indirect) versus pituitary (direct) actions of progesterone and estradiol by a model in which GnRH secretion has been regulated at a set frequency. LH subunit mRNA, intrapituitary LH concentrations and serum LH will be measured to determine the intrapituitary mechanisms of ovarian steroid control of LH secretion. Progesterone binding in the pituitary will be measured to explore the potential role of steroid receptors in the steroid-mediated regulation of LH secretion.
The two main objectives of our study are to 1) Isolate the actions of the ovarian steroids on the pituitary from their actions in the hypothalamus and 2) Determine the effects of estradiol and progesterone on LH subunit mRNA concentrations, pituitary concentrations of LH and LH secretion. In order to examine the effects of ovarian steroids on the pituitary we first had to isolate the pituitary from hypothalamic influences since regulating GnRH secretion would affect pituitary LH synthesis and secretion and steroids are known regulators of GnRH secretion. Isolating the pituitary from hypothalamic influences in vivo is extremely difficult so little is known about distinct steroid action in the pituitary while there is a large amount of information on the combined effects of steroids on the hypothalamus and pituitary. Thus, these studies have provided unique insight into steroid actions in the pituitary.
CHAPTER 3
DIRECT PITUITARY EFFECTS OF ESTRADIOL AND PROGESTERONE ON LUTEINIZING HORMONE RELEASE, STORES AND SUBUNIT MESSENGER RIBONUCLEIC ACIDS

ABSTRACT
To determine the direct, chronic actions of progesterone (P) and estrogen (E) on anterior pituitary synthesis and release of luteinizing hormone (LH), 24 western range ewes underwent hypothalamic-pituitary disconnection (HPD) and ovariectomy (OVX) during the breeding season and were pulsed with exogenous gonadotropin-releasing hormone (GnRH) with or without steroid replacement. Sequential blood samples were collected before infusion of GnRH and on days 7 and 14 of GnRH infusion. P and/or E were implanted subcutaneously on day 7 and remained in place throughout the experiment. Control ewes received only GnRH infusion. Blood sampling was centered around 3 exogenous GnRH pulses. After the final blood sampling, pituitaries were collected and stored at -70°C. Concentrations of LH in serum and pituitaries were determined by radioimmunoassay. Relative concentrations of LH subunit mRNAs were determined by Fast Blot analysis. Simultaneous implantation of P and E lowered LH pulse amplitude 70% and mean serum levels 30% compared to controls. Neither steroid alone affected LH release. E alone or in combination with P lowered LH-β subunit mRNA concentrations 40% compared to
controls while α-subunit levels were unchanged. Only E alone altered the pituitary content of LH, as determined by radioimmunoassay of pituitary homogenates, causing a 60% decrease. We conclude that the combination of P and E is necessary for inhibition of GnRH-stimulated LH secretion. E inhibits GnRH-stimulated LH-β subunit mRNA concentrations but does not affect α-subunit mRNA concentrations. The control of pituitary LH content by P and E is the result of changes in LH-β subunit mRNA concentrations and LH secretion.

INTRODUCTION

The synthesis and tonic release of LH during the luteal phase of the estrous cycle is regulated by the ovaries [Gharib et al., 1990]. The ovarian steroids progesterone and estrogen may alter ovine LH synthesis and secretion through direct action on the pituitary, or indirectly by altering GnRH release from the hypothalamus [Fink G, 1988]. In ovariectomized ewes, chronic treatment with progesterone and estrogen is required to produce normal tonic LH secretion, suggesting a synergistic relationship between these steroids [Karsch et al., 1980]. This synergism is partially attributed to an estradiol-induced increase in the sensitivity of the GnRH pulse generator to progesterone negative feedback [Goodman et al., 1981], causing a reduction in LH pulse
frequency. Ovarian steroids also may play a key role in regulating LH synthesis and storage during the estrous cycle. During the luteal phase of the estrous cycle pituitary concentrations of LH and LH subunit mRNA are at their lowest [Leung et al., 1988]. Progesterone and estradiol probably act in a synergistic fashion to reduce pituitary concentrations of LH and its subunit mRNA [Simard et al., 1988; Corbani et al., 1990]. The role of ovarian steroids in directly modulating pituitary gonadotrope responsiveness to GnRH has not been firmly elucidated because changes in LH synthesis or secretion may reflect altered GnRH release as well as direct steroidal control of pituitary function [Goodman RL, 1988].

Several studies have examined the chronic, direct actions of estradiol on pituitary LH synthesis and secretion. Experimental models include primary pituitary cell cultures, hypogonadal mice, and hypothalamic-pituitary disconnected (HPD) ewes. In female rat and ovine pituitary cell cultures, 24 hour exposure to estradiol enhances the stimulation of LH synthesis and release by GnRH [Huang and Miller, 1980; Ramey et al., 1987]. In the female hypogonadal mouse, chronic exposure to estrogen (2 weeks) prevents the stimulation of LH secretion by exogenous GnRH while augmenting LH synthesis [Saade et al., 1989]. Chronic administration of estradiol (1 week) has no effect on LH synthesis or secretion in short-term (<1 month) ovariectomized HPD ewes infused with exogenous GnRH
[Mercer et al., 1988] while inhibiting the stimulation of LH release by exogenous GnRH in long-term ovariectomized (>6 months) HPD ewes [Clarke et al., 1989]. LH synthesis was not measured in the later study. Progesterone also appears to have direct actions in modulating pituitary synthesis and release of LH, however, prior exposure to estradiol is necessary. In female ovine and rat pituitary cell cultures, progesterone treatment (24 hours) inhibits GnRH-stimulated LH secretion if the cells are pretreated with estradiol [Batra and Miller, 1985; Krey and Kamel, 1990] and chronic progesterone administration (1 week) inhibits the release of LH stimulated by exogenous GnRH in HPD-OVX ewes with prior exposure to estradiol [Clarke et al., 1989], while having no effect in HPD-OVX ewes not treated with estradiol [Clarke and Cummins, 1984]. While considerable attention has been paid to the direct pituitary actions of estradiol and progesterone, relatively little is known about the direct, synergistic regulation of pituitary LH synthesis and release by these hormones.

In this study we examined the role progesterone and estradiol have in directly modulating pituitary synthesis and release of LH. This study was designed to investigate the separate versus combined effects of chronic estradiol and progesterone treatment when a constant frequency and amplitude
of GnRH release is maintained, enabling us to draw conclusions regarding the direct (pituitary) actions of these steroids.

METHODOLOGY AND MATERIALS

The pituitary-hypothalamic interface was disconnected in 24 western range ewes during the breeding season using a transnasal transphenoidal surgical procedure [Clarke et al., 1983], and an aluminum barrier was inserted to prevent reconnection. Four to five days after disconnection ewes were ovariectomized and four to five days following ovariectomy one external jugular vein was cannulated with polyethylene tubing (Intramedic, Clay Adams, Parispanny, NJ). Blood samples were collected at ten minute intervals for four hours after which the cannulae were connected to Tygon tubing (Tygon R-3603, Norton, Akron, OH) using a T-connector (cat. no. N-06365-77, Cole Parmer, Chicago, IL) which allowed infusion and sampling from the same catheter. Infusions were delivered via peristaltic pump (Manostat multichannel cassette pump, New York, NY) which was activated by an electric timer (Chrontrol tabletop timer, Linberg Enterprises, San Diego, CA). LHRH (GnRH) (Sigma Chemical Co., St. Louis, MO) was prepared in sterile saline (0.9%) and stored as a stock solution (10 ug/ml) at 4°C. Working concentrations of GnRH (100 ng/ml) were prepared in saline (100 ml aliquots) and infused every
two hours (bihourly) as a 400 ng/4 ml/3 min pulse. Aliquots of GnRH were changed daily and checked to verify the correct pulse dose and rate.

Ewes were pulsed with GnRH throughout the experiment (14 days). There is a linear increase in LH secreted in response to exogenous GnRH between day 0 and days 5-7 after which LH secretion remains at this maximal level (unpublished observations). On days seven and fourteen blood samples were collected for six hours at ten minute intervals with the sampling periods centered around 3 exogenous GnRH pulses. On day seven, following sample collection, ewes were divided randomly into treatment groups and received either no implants (controls), two 30 mm estrogen implants (E), two 8 x 10 cm progesterone implants (P₄), or two 30 mm estrogen implants plus two 8 x 10 cm progesterone implants (P/E). Estrogen implants were constructed of silastic tubing (Dow Corning Co., Midland, MI) (i.d.=0.132 in, o.d.=0.183 in), packed with β-estradiol (Sigma Chemical Co., St. Louis, MO). Progesterone implants were constructed with non-reinforced 0.005 inch thick silastic sheeting (Dow Corning Co., St. Louis, MO), packed with progesterone (Sigma Chemical Co., St. Louis, MO). Steroid implants, which were placed subcutaneously in the axillary region of the hind limbs, were soaked overnight in water prior to implantation and remained in place the duration of the experiment [Karsch et al., 1987]. Implants raised serum
estradiol concentrations 7.7 ± 1.7 pg/ml (n=6 ewes) and serum progesterone concentrations 2.5 ± 0.4 ng/ml (n=12 ewes) over preimplantation concentrations, as determined by steroid radioimmunoassay. After the last blood sampling, ewes were anaesthetized with sodium pentobarbital and exsanguinated. Separation of the hypothalamus from the pituitary gland was verified by gross inspection. Pituitaries were collected, trimmed free of connective tissue, minced, and immediately frozen in liquid nitrogen for later analysis of LH subunit and GH mRNA concentrations and pituitary content of LH. Serum was harvested from the sequential blood samples and analyzed for LH, estradiol and progesterone concentrations.

Serum LH, estradiol and progesterone were measured by radioimmunoassay as described in appendix 1. Northern blots of LH-β and LH-α subunit mRNA were performed as described in appendix 1. Relative concentrations of LH subunit mRNA were determined as described in appendix 1.

Serum LH profiles for each animal were analyzed with the Cluster Analysis program [Veldhuis and Johnson, 1986; Urban et al., 1988] using a global variance model and the optimal cluster sizes and t-statistics for detecting LH pulses when the sampling interval is 10 minutes [Veldhuis and Johnson, 1988]. Differences in mean LH pulse amplitudes (pulse height minus the preceding nadir), mean LH serum levels and mean LH baselines between days or treatments were determined using
one-way ANOVA and Fisher's least-significant-difference test. Comparisons in treatment means of pituitary concentrations of LH and LH-subunit mRNAs were made using one-way ANOVA and Fisher's least-significant-difference test.

RESULTS

Sequential blood samples were collected before GnRH infusion and 7 and 14 days with pulsatile GnRH infusion. Ewes were treated with either estrogen (E), progesterone (P₄), progesterone and estrogen (P/E) or no steroids (C) for the final 7 days (Fig. 1). Serum LH was non-detectable in HPD-OVX ewes before GnRH infusion and increased to a mean of 1.0 ± 0.1 ng/ml after 14 days of GnRH infusion (p<0.01). The simultaneous implantation of progesterone and estradiol (P/E) reduced LH pulse amplitude 70% in GnRH-treated HPD-OVX ewes compared to pre-steroid treatment values (p<0.01), while neither steroid had any effect on LH pulse amplitude when implanted alone. Mean serum LH profiles of HPD-OVX ewes infused with exogenous GnRH plus subcutaneous progesterone (P₄), estradiol (E), or progesterone and estradiol (P/E₂) implants are shown in Figure 2. HPD-OVX ewes infused with exogenous GnRH receiving no steroid implants served as controls (C). A pulse of LH was associated with each pulse of exogenous GnRH in control HPD-OVX ewes and in HPD-OVX ewes
treated with either steroid alone. LH pulses were noticeably smaller in HPD-OVX ewes treated with both steroids. The mean value for LH pulse amplitude, serum LH concentration and mean LH baseline concentration were determined for each ewe in the final sampling period and the treatment means were compared using one-way ANOVA (Fig. 3). The combination of progesterone and estradiol lowered mean LH pulse amplitude 70% (p<0.01) and mean serum concentration 30% (p<0.05) compared to GnRH-treated controls while the mean LH baseline remained unchanged. Neither steroid had any effect on these three serum LH parameters when implanted alone.

To confirm the hybridization of cDNA to the appropriate size mRNA, total pituitary RNA was separated by electrophoresis and probed for LH–β subunit and α-subunit mRNA (Fig. 4). Base pair lengths of approximately 700 and 900 were determined, respectively. The reported size of LH–β subunit mRNA excluding the poly(A) tail is 550 nucleotides (Virgin et al., 1985) and the α-subunit mRNA is 730 nucleotides excluding the poly(A) tail (Goodwin et al., 1983). To optimize slot blot quantitation of LH subunit mRNA, different RNA concentrations were probed to determine a linear range of hybridization signal when probed for LH subunit mRNA (Fig. 5). From densitometry readings we choose to probe 0.5 ugs of total RNA from different ewes in duplicate for LH subunit mRNA (Fig.
A densitometer was used to quantitate the density of hybridization signals in the slot blots.

At the end of the experiment, following 7 days of steroid treatment with GnRH infusion, the pituitary concentrations of LH and LH subunit mRNAs were determined. Relative α-subunit mRNA concentrations were not affected by any of the steroid treatments and remained at GnRH-infused control concentrations (Fig. 7). Estradiol lowered LH-β subunit mRNA concentrations 40% compared to GnRH-infused controls when implanted alone (p<0.01) or with progesterone (p<0.01). Estradiol treatment led to a 60% reduction in the pituitary content of LH compared to controls (p<0.01) while neither progesterone nor the combination of progesterone and estradiol had any significant effect on pituitary content of LH.

**DISCUSSION**

Based upon this study and several others, we propose that progesterone directly inhibits pituitary LH release if ewes are exposed to estrogen. We report that simultaneous treatment of hypothalamic-pituitary disconnected, ovariectomized (HPD-OVX) ewes with progesterone and estradiol lowers serum LH pulse amplitude when the frequency and amplitude of GnRH release are maintained at constant levels. In a similar study, progesterone reduced LH release in HPD-OVX
ewes infused with exogenous GnRH if the ewes had prior exposure to estradiol [Clarke et al., 1989]. In intact and ovariectomized ewes, direct inhibition of pituitary LH release by progesterone appears dependant upon another ovarian hormone, probably estradiol [Karsch et al., 1977; Wheaton and Mullet, 1982], and progesterone inhibits GnRH-stimulated LH release in sheep and rat pituitary cell cultures only after prior exposure to estradiol [Batra and Miller, 1985; Krey and Kamel, 1990; Batra and Miller, 1986; Krey et al., 1990]. Thus, steroidal control of tonic LH release may be exerted partially through the direct inhibition of pulsatile pituitary LH release by the synergistic action of progesterone and estradiol.

The absence of LH secretion observed in ovariectomized ewes treated chronically with estradiol may be the result of decreased pulsatile GnRH release coupled with a depletion of pituitary stores of LH. In this study we maintained a constant frequency and amplitude of GnRH in the absence of endogenous GnRH release by pulsatile infusion and under these conditions neither progesterone nor estradiol, when implanted alone, had any effect on LH secretion. Similar results have been reported by others [Mercer et al., 1988; Clarke and Cummins, 1984]. It is widely accepted that progesterone alone has no direct effect on pituitary LH release but the role of estradiol in mediating tonic LH release directly at the
pituitary level is not clear. Estradiol can inhibit GnRH pulse amplitude [Caraty et al., 1989] and when GnRH pulse amplitude and frequency are not controlled, estradiol decreases LH pulse amplitude [Goodman and Karsch, 1980]. Thus, in ovariectomized ewes, estradiol may inhibit LH pulse amplitude indirectly via the hypothalamus. Although we did not observe a decrease in LH pulse amplitude with estradiol treatment we detected a partial reduction in the pituitary stores of LH. We speculate that continued exposure to estradiol would have depleted intracellular stores of LH. This direct action of estradiol on pituitary LH content could contribute to the reduction in LH pulse amplitude observed in ovariectomized ewes treated chronically with estradiol for two weeks [Nilson et al., 1983]. Thus, in addition to decreasing GnRH release, estradiol inhibition of LH pulse amplitude in ovariectomized ewes may also be mediated directly at the pituitary through regulation of pituitary stores of LH.

To interpret the physiological role of ovarian steroids from studies with ovariectomized animals the dose and administration of the steroids must be taken into account. Our aim in this study was to mimic a luteal phase steroid signal using luteal phase concentrations of progesterone and estradiol for a prolonged period. However, the estradiol concentration reached a follicular phase concentration of estradiol (approximately 8 pg/ml). Whether a prolonged
follicular phase concentration of estradiol affects pituitary LH release differently than a prolonged luteal phase concentration of estradiol is unknown. Interpreting the physiological role of progesterone from these studies is simpler because the sustained progesterone concentration used in this study closely resembles the progesterone signal during the luteal phase.

Estradiol inhibition of α-subunit mRNA concentrations appears to be mediated indirectly through altered hypothalamic activity while inhibition of LH-β subunit mRNA concentrations may be mediated at both hypothalamic and pituitary levels. In this study using HPD-OVX ewes receiving GnRH infusions, chronic treatment with estradiol alone or in combination with progesterone reduced LH-β subunit mRNA concentrations but did not affect α-subunit mRNA concentrations. In contrast, progesterone treatment alone had no effect on α-subunit or LH-β subunit mRNA levels. Thus, the reduction in GnRH-stimulated LH-β subunit mRNA levels observed in the presence of progesterone and estradiol may be due solely to the inhibitory action of estradiol since progesterone had no additive effect on LH-β subunit mRNA. In ovariectomized ewes and rats, estradiol lowered α-subunit and LH-β subunit mRNA concentrations greater than 90% compared to nontreated ovariectomized animals [Simard et al., 1988; Nilson et al., 1983; Gharib et al., 1986] and this effect may be attributed
to the integrated negative feedback effects of estradiol at the hypothalamic and pituitary levels [Nett et al., 1990]. In our study using hypothalamic-pituitary disconnected ewes we have isolated the direct pituitary actions of estradiol. It appears that estradiol inhibition of α-subunit mRNA concentrations in gonadectomized animals is primarily mediated indirectly, possibly through a decrease in GnRH pulse frequency and amplitude. In addition, because we observed an estradiol-induced decrease in GnRH-stimulated LH-β subunit mRNA levels to only 40% of GnRH-treated control values, the reduction of LH-β subunit mRNA concentration by estradiol in gonadectomized animals probably is mediated at both hypothalamic and pituitary levels.

The length of time between ovariectomy and subsequent estradiol exposure may alter the effect of estradiol on LH synthesis and release. There are discrepancies in the literature concerning the direct effects of estradiol on LH-subunit mRNA levels and LH secretion in HPD-OVX ewes receiving GnRH infusions. In this and another report using HPD ewes ovariectomized for one month or less, chronic administration of estradiol had no effect on LH pulse amplitude [Mercer et al., 1988]. In contrast, Clarke et al. demonstrated that chronic estradiol treatment lowered LH pulse amplitude in ewes ovariectomized 6 months prior to study [Clarke et al., 1989]. Because both reports have employed
subcutaneous implantation of one 30 mm silastic estradiol implant for one week, it is likely that the length of time post-ovariectomy, prior to steroid implantation, may lead to varying results. Perhaps a loss of steroid receptors occurs following ovariectomy altering the function of steroid hormones [Schanbacher et al., 1984]. Newly discovered ovarian proteins [Busbridge and Chamberlain, 1990] and as yet unidentified follicular fluid proteins [Lumpkin et al., 1984] may also have a role in the action of estradiol. A similar explanation may account for the dissimilar reports of estradiol's effect on LH-β subunit mRNA concentrations in HPD-OVX ewes [Mercer et al., 1988].

The pool of intracellular LH may reflect changes in LH-β subunit mRNA levels and LH secretion. Pituitary concentrations of LH-β subunit mRNA have been correlated with pituitary content of LH [Nilson et al., 1983; Leung et al., 1987; Godine et al., 1980; Grotjan and Leveque, 1984; Lalloz et al., 1988]. Several investigators have hypothesized that changes in pituitary content of LH may be the summation of changes in LH subunit mRNA concentrations and LH secretion [Saade et al., 1989; Kato et al., 1989; Abbot et al., 1988]. Two examples addressing this level of control of pituitary LH content are apparent in this study. When progesterone and estradiol were both present, LH secretion was reduced and LH-β subunit mRNA levels were lowered, subsequently, no change in
the pituitary content of LH was observed because the reduction in secretion of LH from the pituitary was matched by a reduction in LH synthesis. When only estradiol was present, LH secretion was unaffected while LH-β subunit mRNA concentrations were lowered, leading to a decrease in the pituitary content of LH. In this case there was no alteration in the output of LH from the pituitary pool while synthesis of LH was reduced which led to a depletion of pituitary LH stores. It appears that steroidal control of LH synthesis and secretion may be dissociated [Mercer, 1990]. A possible mechanism may be the steroid-induced synthesis of protein intermediaries that specifically block intracellular signaling pathways or alter the pool of LH [Krey and Kamel, 1990]. Further studies are needed to examine these speculative mechanisms.

In summary, we have determined that chronic, simultaneous implantation of progesterone and estradiol will inhibit the stimulation of LH release by exogenous GnRH in HPD-O VX ewes, while neither steroid alone has any direct effects on pituitary LH release. In this study we have also determined that chronic estradiol administration will inhibit GnRH-stimulated LH-β subunit mRNA concentrations without affecting α-subunit mRNA levels. The distinct regulation of LH-β subunit mRNA concentrations and LH pulsatile release by
ovarian steroids results in a concomitant change in the pituitary content of LH.
Fig. 1. Mean LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and after steroid treatment. HPD-OVX ewes were infused with exogenous GnRH for 14 days (controls) with either progesterone and estradiol (P/E), estradiol (E) or progesterone P treatment for the final 7 days. Values for LH pulse amplitude represent the mean of 3 pulses from 6 HPD-OVX ewes (mean ± SEM). Bars with different letters are significantly different (p<0.01; n=6). P and E, in combination, lowered mean LH pulse amplitude 70% compared to pre-treatment values.
Fig. 2. Mean serum LH profiles (±SEM) of HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. HPD-OVX ewes (n=6 ewes/treatment) were infused with exogenous GnRH for 14 days (C) and treated with either progesterone (P), estradiol (E), or progesterone and estradiol (P/E) for the final 7 days. Arrows signify exogenous GnRH pulse infusions.
Fig. 3. Mean values for LH pulse amplitude, serum LH concentration and baseline LH concentration in HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. HPD-OVX ewes were infused with exogenous GnRH for 14 days (controls) plus progesterone and estradiol (P/E), estradiol (E) or progesterone P treatment for the final 7 days. Bars with different letters are significantly different between treatments (p<0.01; n=6). Only the combination of P and E lowered mean LH pulse amplitude and serum LH concentration compared to controls.
Figure 4: Photographs of autoradiograms confirming identity of mRNA being examined. Four lanes of total pituitary RNA (4 μg/lane) from an ovariectomized ewe was separated by electrophoresis and probed for LH-β subunit or LH-α subunit mRNA. The migration of RNA standards is shown alongside the photograph. The length of LH-β subunit and α-subunit mRNA were approximately 700 and 900 basepairs, respectively.
Figure 5: Photographs of autoradiograms of slot blots determining linear range. 0.1, 0.25, 0.5, 0.75, 1.0 and 1.5 ug of total pituitary RNA from 4 ovariectomized ewes were immobilized onto nylon membranes with a slot blot apparatus and probed for LH-β subunit mRNA or α-subunit mRNA and then reprobed for GH mRNA. Each vertical series of slots has total RNA from two animals for a total of 4 ewes on each blot. Ewe 1 is in the upper left portion of the blot, ewe 2 is in the upper right portion, ewe 3 in the lower left portion of the blot and ewe 4 in the lower right portion. After probing for LH-β or LH-α subunit the blots were reprobed for GH mRNA and the corresponding GH blots are shown next to their respective LH subunit mRNA blots. The sequence of total RNA concentrations are shown alongside the photographs although not exactly aligned with the RNA bands.
<table>
<thead>
<tr>
<th>LH-β TOTAL GH</th>
<th>LH-α TOTAL GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA 1</td>
<td>RNA 1</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

3 4 3 4

3 4 3 4
Figure 6: Average density of hybridization signals of 0.1 to 1.5 ug of total pituitary RNA probed for LH-β subunit, LH-α subunit or GH mRNA. The blots shown in the previous figure were scanned with a densitometer and the density of the mRNA bands at each concentration of total RNA (0.1, 0.25, 0.5, 0.75, 1.0, and 1.5) for the 4 ewes was determined for the three different mRNA's and plotted as a function of total RNA concentration. There was a linear increase in the densities of the hybridization signals from 0.1 to 1.0 ugs of total RNA for all three mRNA's although LH-α subunit mRNA appeared to level off slightly at the upper end of this range.
Figure 7: Photographs of autoradiograms of slot blots determining relative levels of LH-β subunit, α-subunit and GH mRNA. Total pituitary RNA (0.5 ug/slot) from 12 ewes with 4 different treatments were probed in duplicate for LH-β subunit or α-subunit mRNA and reprobed for GH mRNA. Each horizontal series of bands represents duplicate loadings of total pituitary RNA from a different animal with a particular steroid treatment, for a total of 12 ewes per blot. After probing for LH-β or LH-α subunit the blots were reprobed for GH mRNA and the corresponding GH blots are shown next to their respective LH subunit mRNA blots. The sequence of treatments corresponding to RNA bands in the photograph are shown alongside the photograph although not exactly aligned.
Fig. 8. Relative α-subunit mRNA, LH-β subunit mRNA and LH tissue concentrations in HPD-OVX ewes infused with exogenous GnRH with or without 7 days of steroid treatment. Relative LH subunit mRNA concentrations and pituitary concentrations of LH were determined in HPD-OVX ewes infused with exogenous GnRH for 14 days (control) plus progesterone and estradiol (P/E), estradiol (E) or progesterone P treatment for the final 7 days. Bars with different letters are significantly different (p<0.01; n=6). E reduced the relative concentration of LH-β subunit mRNA alone, or with P, but did not affect the relative α-subunit mRNA concentration, compared to controls. Treatment with E₂ alone significantly reduced the pituitary content of LH.
CHAPTER 4

PROGESTERONE DIRECTLY INHIBITS PITUITARY LUTEINIZING HORMONE SECRETION IN AN ESTRADIOL-DEPENDENT MANNER

ABSTRACT

Progesterone (P) and estradiol (E) inhibit pituitary luteinizing hormone (LH) secretion in a synergistic fashion. This study examines the endocrine nature of this interaction. Nine ovariectomized (OVX) ewes underwent hypothalamic-pituitary disconnection (HPD) and were infused with 400 ng GnRH every 2 hours throughout the experiment. After 7 days of infusion, E was implanted subcutaneously. Four days later E implants were exchanged for blank implants in 4 ewes and P implants in 5 ewes. These implants remained in place another 4 days. Blood samples were collected around exogenous GnRH pulses before and 0.5 to 96 hours after implant insertion and exchange. LH and P concentrations were determined in serum by RIA. One month later 4 of the HPD-OVX ewes previously implanted with steroids were reinfused with GnRH and the implantation protocol was repeated using blank implants only. In estradiol-primed ewes, P significantly lowered LH secretion after 12 hours of implantation and LH secretion remained inhibited through day 4 (p<0.05). Removing E transiently lowered LH secretion and this effect was significant only 24 hours after E withdrawal (p<0.05). These data suggest that P has a direct, E-dependent inhibitory effect on pituitary LH.
release and E may sustain pituitary gonadotrope response to
GnRH.

INTRODUCTION

Tonic secretion of luteinizing hormone (LH) is inhibited
by the ovaries during the luteal phase of the estrous cycle
[Goodman RL, 1988]. This inhibition is due to the ovarian
steroids progesterone and estradiol [Karsch et al., 1980;
Goodman et al., 1981]. Ovarian steroids may suppress LH
secretion indirectly by lowering gonadotropin-releasing
hormone (GnRH) secretion from the hypothalamus or directly by
decreasing gonadotroph response to GnRH [Gharib et al., 1990].
During the luteal phase, estradiol increases the sensitivity
of the GnRH pulse generator to progesterone negative feedback,
thereby reducing LH secretion [Karsch FJ, 1987]. The actions
of progesterone and estradiol on the pituitary during the
luteal phase remain ambiguous.

Progesterone and estradiol directly inhibit secretion of
LH from the pituitary in a synergistic fashion [Girmus and
Wise, 1991; Clarke et al., 1989]. Progesterone and estradiol
receptors are present in gonadotrophs, making these cells
potentially responsive to steroid action [Sprangers et al.,
1989; Fox et al., 1990]. In intact and ovariectomized ewes,
progesterone appears to inhibit pituitary LH secretion in an
estradiol-dependent manner [Karsch et al., 1977; Wheaton and Mullet, 1982] and estradiol is required for inhibition of GnRH-stimulated LH secretion by progesterone in both sheep and rat pituitary cell cultures [Batra and Miller, 1985; Batra and Miller, 1986; Krey and Kamel, 1990; Krey et al., 1990]. The present study begins to delineate the endocrine mechanisms of progesterone inhibition of pituitary LH secretion and further examines the direct versus indirect actions of ovarian steroid regulation of LH secretion. This study will determine if progesterone inhibits pituitary LH secretion in hypothalamic-pituitary disconnected ewes primed with estradiol and determine the time course of progesterone inhibition of LH secretion.

METHODS AND MATERIALS

The hypothalamic-pituitary interface was surgically disconnected (HPD) in nine ovariectomized (OVX) western range ewes and jugular infusions of 400 ng GnRH in 4 ml saline delivered over 2 min every 2 hours were initiated and continued throughout the experiment [Girmus and Wise, 1991]. After 7 days of infusion HPD-OVX ewes were implanted subcutaneously with estradiol. The estradiol implants were removed after 4 days and 5 of the ewes received progesterone implants and 4 ewes received blank implants. Steroid
Implants were constructed as described previously to raise serum estradiol concentrations approximately 8 pg/ml and serum progesterone concentrations approximately 2 ng/ml over preimplantation values [Girmus and Wise, 1991]. One month later in a subsequent experiment, 4 of the HPD-OVX ewes were reinfused with the GnRH regimen and blank implants were inserted for 4 days, removed and new blanks implanted for 4 days, mimicking the steroid implantation protocol. These ewes served as controls.

To measure LH release in response to exogenous GnRH, blood samples were collected 20 and/or 10 minutes before and 0, 10, 20, 30, 40, 50, and 60 minutes after an exogenous GnRH pulse. Blood samples were collected around exogenous GnRH pulses before and from 0.5 to 96 hours after the insertion and/or exchange of steroids or blanks. Serum was harvested and stored at -20°C until assayed for LH and progesterone by RIA.

Concentrations of LH and progesterone in serum were measured as described in appendix 1.

Values for LH pulse amplitude (pulse height minus the preceding nadir) in individual ewes were determined and mean values ±SEM for pulse amplitude at different times for each treatment were calculated. Statistical comparisons between treatments and over time were made with repeated measures ANOVA and Fisher's least-significance-difference tests.
Correlative comparisons were made with regression analysis and curves were calculated using their least-squares-fit.

**RESULTS**

To ensure that exchanging subcutaneous silastic implants had no effect on LH secretion we measured serum LH before and after blank implants were exchanged. Inserting and removing subcutaneous blank implants had no effect on the stimulation of LH secretion by exogenous GnRH (Fig. 5). We also determined the effect of estradiol-priming on LH secretion in this model. Implanting estradiol appeared to enhance slightly the stimulation of LH secretion by exogenous GnRH but this effect did not reach statistical significance (Fig. 6). However, withdrawal of the estradiol implants caused a partial, transient drop in LH secretion that was significant only 24 hours after estradiol removal (p<0.05; Fig. 7).

To determine the direct effects of progesterone on LH secretion in estradiol-primed HPD-OVX ewes we measured LH secretion before and after removing estradiol implants and inserting progesterone implants. Twelve hours after implanting progesterone following estradiol removal LH secretion was significantly reduced and LH secretion remained inhibited through day 4 (p<0.05; Fig. 8). Mean LH pulse amplitude was significantly lower from 48 to 96 hours post-
implantation in estradiol-primed ewes treated with progesterone compared to estradiol-primed ewes implanted with blanks (p<0.05; Fig. 9). Plotting LH pulse amplitude versus the time post-progesterone implantation revealed no lag time in the reduction of LH secretion associated with progesterone implantation (Fig. 10). Serum progesterone concentrations rose from non-detectable levels before implantation to a maximum of 1.8 ng/ml 8 hours after progesterone implantation (Fig. 11A). The rise in progesterone levels was associated with a corresponding decline in LH pulse amplitude and there was a strong inverse correlation between serum progesterone concentrations and LH pulse amplitude (r=0.98; p<0.01; Fig. 11B).

DISCUSSION

These data demonstrate that progesterone directly inhibits pituitary LH release in an estradiol-dependent manner. We recently reported that progesterone and estradiol together but not separately, inhibit pituitary LH secretion in HPD-OVX ewes infused with exogenous GnRH [Girmus and Wise, 1991]. The current study demonstrates that progesterone will directly inhibit pituitary LH release in HPD-OVX ewes primed with estradiol. This supports other studies with intact, OVX or HPD-OVX ewes in which progesterone inhibition of LH
secretion is dependent upon estradiol exposure [Clarke et al., 1989; Karsch et al., 1977; Wheaton and Mullet, 1982]. In addition, progesterone inhibits GnRH-stimulated LH secretion in cultured sheep and rat pituitary cells only with prior exposure to estradiol [Batra and Miller, 1985; Batra and Miller, 1986; Krey and Kamel, 1990; Krey et al., 1990].

Ovarian steroid inhibition of tonic LH release during the luteal phase may be the result of direct (pituitary) and indirect (hypothalamic) regulation of LH secretion. Treatment with both progesterone and estradiol is required for normal tonic LH secretion in ovariectomized ewes [Goodman RL, 1988]. The release of GnRH into the portal circulation is inhibited by estradiol and progesterone [Karsch et al., 1987]. In our study progesterone directly inhibits pituitary LH release in estradiol-primed HPD-OVX ewes infused with exogenous GnRH pulses. Thus, we propose two contributing endocrine mechanisms for synergistic inhibition of LH release by the steroid hormones: 1) estradiol sensitization of the hypothalamus to progesterone negative feedback [Karsch et al., 1980], and 2) estradiol priming of the pituitary which acts to sensitize the pituitary to direct progesterone inhibition. Having two physiological sites of regulation may allow for a more fine-tuned control of LH secretion during the estrous cycle.
Progesterone may not suppress pituitary LH secretion by inhibiting pituitary LH synthesis. Inhibition of LH secretion by progesterone and estradiol occurs without affecting pituitary concentrations of LH or LH subunit mRNA [Girmus and Wise, 1991]. In addition, a one to two hour delay between synthesis and secretion of LH has been predicted [Phillips et al., 1988; Ruddon et al., 1981] and such a lag time would be expected if progesterone reduced LH secretion by lowering LH synthesis. No lag time was observed following progesterone implantation in this study, suggesting progesterone inhibits pituitary LH secretion independent of changes in LH synthesis. The rapid reduction in serum LH observed after progesterone treatment may be due to regulation of LH release at a plasma membrane site [Schumacher M, 1990; Ke and Ramirez, 1990; Klangkalya and Chan, 1987] or via a rapid genomic action [Spelsberg et al., 1987] which regulates protein components critical to the gonadotrope signaling-secretory pathway [Krey and Kamel, 1990].

The stimulatory phase of estradiol regulation of LH secretion in ovariectomized ewes may be due to its direct effects on the pituitary. In this study estradiol appears to maintain the pituitary responsiveness to GnRH. The effect was subtly apparent after implanting estradiol and following estradiol withdrawal when LH pulse amplitude was transiently lowered. This effect was noticeable 4 days after estradiol
treatment, however, 7 days of treatment with estradiol has no direct effect on LH secretion [Girmus and Wise, 1991]. In the ovariectomized ewe, chronic estradiol treatment has a triphasic action on LH secretion: inhibitory, stimulatory and then inhibitory again [Herring et al., 1991]. In the present study the effect of estradiol resembles a stimulatory phase action, possibly the result of an up-regulation of GnRH receptors [Gregg and Nett, 1989; Sealfon et al., 1990]. Alternatively, estradiol may affect cell-cell interactions in the pituitary to stimulate gonadotrope secretion of LH in a paracrine fashion [O'Halloran et al., 1991; Baes et al., 1987; Shirasawa et al., 1983].

We routinely measure serum LH pulse amplitudes in HPD-OVX ewes infused with GnRH that are three-quarters of that measured in ovariectomized ewes using our RIA. The low pulse amplitudes may be the result of inadequate GnRH stimulus or disruption of pituitary homeostasis by surgical disconnection. Exogenous GnRH infusion may not effectively mimic endogenous GnRH release, thus resultant pulsatile release of LH becomes low. The normal GnRH signal is a rapid, dramatic release of GnRH into the portal capillary bed; maximum pulse height occurs within 30 seconds of the onset of secretion [Moenter and Karsch, 1991]. Our infusion of GnRH via jugular catheter occurs over a two minute period and may be quickly diluted in the general circulation. Changing the contour of the GnRH
pulse affects the shape of the LH pulse; rounding off the GnRH pulse rounds off the LH pulse, effectively lowering the height of the LH pulse [Handelsman et al., 1988]. Alternatively, the surgical disconnection might affect autocrine/paracrine intrapituitary actions required for optimal secretion of LH by pituitary gonadotropes.

In summary, progesterone directly inhibits secretion of LH from the pituitary in an estradiol-dependent manner, suggesting that inhibition of tonic release of LH by the ovaries during the luteal phase of the estrous cycle is due partially to the direct, synergistic inhibition of pituitary LH secretion by progesterone and estradiol. Progesterone inhibition of pituitary LH secretion is rapid and not associated with changes in LH synthesis, hence progesterone may specifically block LH secretion through a novel and undefined intracellular mechanism. Finally, estradiol appears to sustain the pituitary gonadotrope response to GnRH, thus estradiol stimulation of LH secretion in ovariectomized ewes may be due to its direct effects on the pituitary.
Figure 9: The mean (±SEM) serum LH profile of HPD-OVX ewes infused with exogenous GnRH before and 12 to 48 hours after exchanging blank implants (n=4). Arrows represent exogenous GnRH pulses. Inserting and removing blank implants had no effect on GnRH-stimulated LH release.
Figure 10: The mean (±SEM) serum LH profile of HPD-OVX ewes infused with exogenous GnRH before and 12 to 96 hours after estradiol implantation (n=4). Arrows represent exogenous GnRH pulses. Implanting estradiol had no significant effect on LH release.
Figure 11: The mean (±SEM) serum LH profile of estradiol-treated HPD-OVX ewes before and 0.5 to 96 hours after estradiol withdrawal (n=4). Arrows represent exogenous GnRH pulses. Removing estradiol implants transiently lowered LH release (p<0.05).
Figure 12: The mean (±SEM) serum LH profile of estradiol-treated HPD-OVX ewes before and 0.5 to 96 hours after estradiol withdrawal and progesterone implantation (n=5). Arrows represent exogenous GnRH pulses. Progesterone significantly reduced LH release from 8 to 96 hours post-implantation (p<0.01).
Figure 13: Mean (±SEM) values for LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and 0.5 to 96 hours after various steroid treatments (n=4-5 ewes/treatment). Bars with stars are significantly different at their respective time points (p<0.05). LH secretion in HPD-OVX ewes primed with estradiol was lowered significantly by progesterone treatment compared to ewes receiving blanks after estradiol priming (p<0.01).
Figure 14: Mean (±SEM) values for LH pulse amplitude were plotted against time and the least-squares fit curve determined in HPD-OVX ewes infused with exogenous GnRH, primed with estradiol and treated with blank or progesterone implants. No lag time was observed in the reduction of LH secretion caused by estradiol removal or progesterone implantation.
Figure 15: Mean (±SEM) values for serum progesterone and LH pulse amplitude were determined before and 0.5 to 24 hours after progesterone implantation in estradiol-primed HPD-OVX ewes infused with exogenous GnRH (n=4). A. The rise in progesterone concentrations from 0 to 8 hours inversely matched the decline in LH pulse amplitude during this time. B. There was a strong inverse correlation between serum progesterone concentrations and mean values for LH pulse amplitude (r=-0.98; p<0.01).
CHAPTER 5
PROGESTERONE BINDING ASSOCIATED WITH DIRECT,
ESTRADIOL-DEPENDENT INHIBITION OF LH SECRETION
BY PROGESTERONE

ABSTRACT

In the ewe, progesterone P inhibits pituitary luteinizing hormone (LH) secretion but only in ewes with prior exposure to estradiol. This study examines the endocrine and cellular nature of this action. Sixteen ovariectomized ewes underwent hypothalamic-pituitary disconnection (HPD) and were infused with 400 ng gonadotropin-releasing hormone (GnRH) every 2 hours throughout the experiment. Ewes were divided randomly into 4 groups of 4 ewes each. All groups except controls received estradiol (E) implants on day 6 of GnRH infusion. One group received P implants in addition to E on day 10. A second group received only E throughout the experiment (through day 11). In a third group, E implants were removed and exchanged for P implants on day 10. The fourth group received no steroid treatments and served as a control. Blood samples were collected around exogenous GnRH pulses on day 10 before P implant insertion and/or exchange and one day later (day 11). After the second blood sampling, ewes were exsanguinated, pituitaries collected and total cytosolic P binding capacity was determined by radioreceptor assay. Serum LH concentrations were determined by radioimmunoassay. P
reduced LH secretion by 55% (p<0.05) in both groups treated with estradiol. E raised P binding capacity 4-fold compared to controls (p<0.05) and P raised P binding capacity 8-fold in E-treated ewes compared to controls (p<0.05). These data suggest that P directly inhibits pituitary LH secretion in an E-dependent manner and this effect is associated with enhanced P binding in the pituitary.

**INTRODUCTION**

Ovarian steroid inhibition of tonic LH release during the luteal phase of the estrous cycle is the result of direct (pituitary) and indirect (hypothalamic) regulation of LH secretion (Girmus and Wise, in press). In the hypothalamus, estradiol reduces LH secretion by increasing the sensitivity of the GnRH pulse generator to progesterone negative feedback (Karsch FJ, 1987), possibly due to an up-regulation of progesterone receptors in the hypothalamus by estradiol (Lauber et al., 1991).

Direct inhibition of pituitary LH secretion in vivo by progesterone and estradiol occurs in a synergistic fashion; estradiol is required for progesterone inhibition of LH secretion (Girmus and Wise, 1991, Clarke et al., 1989). Neither steroid alone inhibits pituitary LH secretion directly, but progesterone inhibits LH secretion if the
pituitary is primed with estradiol (Girmus and Wise, in press). Several in vitro studies support this in vivo observation (Batra and Miller, 1985; Batra and Miller, 1986; Krey and Kamel, 1990; Krey et al., 1990).

Those observations suggest that the dependence of progesterone on estradiol may be due to a requisite up-regulation of progesterone receptors. Furthermore, progesterone and estradiol receptors are present in gonadotrophs (Sprangers et al., 1989; Fox et al., 1990) and estradiol up-regulation of pituitary progesterone receptor concentrations in vitro is associated with decreased LH secretion (Krey et al., 1990).

This study examines the regulation of progesterone binding in the pituitary associated with ovarian-steroid mediated inhibition of pituitary LH secretion. Hypothalamic-pituitary disconnected ewes infused with a constant frequency of GnRH boluses will be used in this study to specifically examine steroid action on the pituitary and distinguish pituitary versus hypothalamic effects of ovarian steroids.

METHODS AND MATERIALS

The hypothalamic-pituitary interface was surgically disconnected (HPD) in sixteen ovariectomized (OVX) western range ewes and jugular infusions of 400 ng GnRH in 4 ml saline
delivered over 2 min every 2 hours were initiated and continued throughout the experiment [Girmus and Wise, 1991]. Ewes were divided randomly into 4 groups of 4 ewes. One group served as controls and received no steroid treatment (C). The other 3 groups were implanted subcutaneously with estradiol after 6 days of GnRH infusion (day 6). On day 10 estradiol implants were removed in one group and replaced with progesterone implants for 1 day (E-P). Another group received progesterone implants on day 10 but the estradiol implants were not removed (E+P). In the third group estradiol implants remained in place through day 11 (E). Steroid implants were constructed as described previously and one 30 mm estradiol implant was used to raise serum estradiol concentrations approximately 3-4 pg/ml and serum progesterone concentrations are raised approximately 2 ng/ml over pre-implantation values with this implantation protocol (Girmus and Wise, 1991).

To measure LH release in response to exogenous GnRH, blood samples were collected 20 and 10 minutes before and immediately after an exogenous GnRH pulse and 10, 20, 30, 40, 50, and 60 minutes after an exogenous GnRH pulse. Blood samples were collected around exogenous GnRH pulses on day 10 before progesterone was implanted and one day after progesterone was implanted (day 11). Serum was harvested and stored at -20°C until assayed for LH by RIA.
Concentrations of LH in serum were measured as described in appendix 1 and progesterone binding capacity was determined as described in appendix 1. To initially verify measurement of progesterone binding, Scatchard and saturation analysis of pituitary cytosolic protein preparations were performed.

Values for LH pulse amplitude (pulse height minus the preceding nadir) in individual ewes were determined and mean values ±SEM for pulse amplitude at different times for each treatment were calculated. Statistical comparisons of LH pulse amplitudes on day 10 versus day 11 were made with one-way ANOVA.

After collecting blood samples on day 11 ewes were anaesthetized with sodium pentobarbital and exsanguinated. Separation of the hypothalamus from the pituitary was verified by gross inspection. Pituitaries were collected, trimmed free of connective tissue, minced and suspended immediately in ice-cold TG buffer for subsequent analysis (within 1 hour) of progesterone receptor binding capacity.

Comparisons of total cytosolic pituitary progesterone receptor binding capacities between treatments were made with one-way ANOVA.
RESULTS

To determine the direct effects of progesterone on LH secretion in ewes primed with estradiol we measured LH secretion in ewes treated with estradiol and progesterone (E+P) or estradiol followed by progesterone (E-P) before (day 10) and after (day 11) progesterone implantation (Fig. 12). Progesterone implantation for 1 day decreased LH secretion by 55% in both groups treated with estradiol (p<0.05). Mean serum LH profiles on day 11 are shown in Figure 13. LH pulses are noticeably smaller in ewes treated with progesterone compared to controls while estradiol treatment alone had no apparent effect on LH pulse height.

The influence of steroid treatment on progesterone binding in the pituitary was determined using a cytosolic progesterone receptor assay. Progesterone receptor binding capacity was raised 4-fold (p<0.01) by estradiol treatment compared to controls and 8-fold (p<0.01) when progesterone was combined with estradiol priming or treatment (Fig. 15).

DISCUSSION

Our current study confirms that progesterone directly inhibits pituitary LH secretion in estradiol-primed hypothalamic-pituitary disconnected ewes. In this study progesterone inhibited pituitary LH secretion in hypothalamic-
pituitary disconnected, ovariectomized ewes primed with estradiol and infused with a constant rate of GnRH boluses. Several *in vivo* and *in vitro* studies support this finding (Karsch et al., 1977; Wheaton and Mullett, 1982; Batra and Miller, 1985; Batra and Miller, 1986; Clarke et al., 1989; Krey and Kamel, 1990; Krey et al., 1990) and other studies have demonstrated estradiol-dependent inhibition of hypothalamic GnRH secretion by progesterone (Karsch et al., 1980; Karsch et al., 1987). Thus, progesterone inhibition of LH secretion during the luteal phase of the estrous cycle in the ewe may occur via pituitary and hypothalamic mechanisms. Alternatively, if changes in GnRH pulse frequency affect steroid action on the pituitary, our studies using only one pulse frequency may not accurately address the role of the hypothalamus in steroid-induced LH suppression.

The direct effect of estradiol on pituitary LH secretion may be dose-dependent. In this study estradiol did not affect pituitary LH secretion. We reported previously that estradiol can maintain pituitary LH secretion (Girmus and Wise, in press). In the previous study, the dose of estradiol raised serum estradiol levels approximately 6-8 pg/ml; in this study one-half of that dose was used (approximately 3-4 pg/ml). Perhaps the higher dose of estradiol, which approximates a high follicular phase concentration of estradiol, is stimulatory and the lower dose of estradiol, which would more
closely approximate a luteal phase concentration of estradiol has no direct effect on LH secretion.

The inhibition of pituitary LH secretion by progesterone may be associated with the enhancement of progesterone binding by estradiol. In this study estradiol increased cytosolic progesterone binding capacity 4-fold compared to nearly nondetectable levels in controls. Negligible progesterone binding in untreated, ovariectomized ewes may render LH secretion in these ewes insensitive to progesterone inhibition as observed in several studies (Clarke et al., 1989; Girmus and Wise, 1991)). With estradiol priming sufficient progesterone binding occurs and progesterone gains its inhibitory action.

Progesterone may enhance its own binding in the pituitary in estradiol-primed ewes. In this study, a 2-fold increase in progesterone binding was observed in the estradiol-primed ewes treated with progesterone compared to ewes treated with estradiol alone. We predict that progesterone has this effect only in estradiol-treated ewes since progesterone has no direct action on the pituitary in ewes not treated with estradiol (Girmus and Wise, 1991; Clarke et al., 1989). This is a unique report of enhancement of progesterone binding in the pituitary. There are confirming reports of up-regulation of progesterone receptors in certain uterine tissues (Lessey et al., 1988; Clarke CL, 1990). Pituitary progesterone
receptors were homologously down-regulated in estradiol-treated monkeys implanted simultaneously with progesterone for 14 days (Sprangers et al., 1990) and we predict that continued exposure to progesterone would down-regulate the progesterone receptor.

The enhanced binding of progesterone in the pituitary could be due to increased progesterone receptor binding or increased binding by other proteins that bind progesterone. Corticoid receptors and corticoid-binding globulins bind progesterone and there may be other unidentified proteins that bind progesterone (Kato J, 1985) all of which are potentially regulated by estradiol.

The mechanism whereby progesterone directly inhibits pituitary LH secretion is unknown. Progesterone lowers GnRH receptor concentration (Laws et al., 1990) and GnRH receptor mRNA concentration (Sealfon et al., 1990) and this is associated with reduced LH secretion. However, other mechanisms may be involved (Wise et al., 1984). Progesterone effects on LH secretion appear to require ribonucleic acid and protein synthesis (Turgeon and Waring 1991; Kammel and Krey, 1990) and could involve regulation of G-protein concentrations (Davidson et al., 1991; Bouvier et al., 1991).

In summary, progesterone directly inhibits pituitary LH secretion in an estradiol-dependent manner. The dependence on estradiol is associated with enhanced binding of progesterone
in the pituitary. Progesterone further increases its own binding in the pituitary in estradiol-primed ewes. We propose that estradiol increases pituitary progesterone binding and progesterone further enhances its own binding to inhibit pituitary LH secretion.
Figure 16: Mean (±SEM) values for LH pulse amplitude in HPD-OVX ewes infused with exogenous GnRH before and after steroid treatment. Controls received no steroids (C) and the other 3 groups received E implants on day 6. One group only received the E implants (E); one group received E implants and P was implanted with E on day 10 (E+P); the final group received E implants and E was removed and replaced with P on day 10 (E–P). P reduced mean LH pulse amplitude by 55% in both groups treated with E (p<0.05).
Figure 17: Mean (±SEM) serum LH profiles of HPD-OVX ewes infused with exogenous GnRH and treated with steroids. Serum LH profiles from day 11 for the 4 different groups described in the legend for figure 15 are shown.
Figure 18: Pituitary cytosolic progesterone receptor binding capacity in HPD-OVX ewes infused with exogenous GnRH and treated with steroids. Mean (±SEM) values for total pituitary cytosolic progesterone binding capacity was determined in the 4 groups of ewes described in the legend of figure 12. E raised P receptor binding capacity 4-fold (p<0.05) and P raised P receptor binding capacity 8-fold in E-treated ewes (p<0.05), compared to controls (n=4 ewes/treatment).
CHAPTER 6

SUMMARY

Ovarian steroid inhibition of tonic LH release during the luteal phase of the estrous cycle may be partially due to the direct inhibition of pituitary LH secretion by progesterone. These studies demonstrate that progesterone and estradiol can directly inhibit pituitary LH secretion in a synergistic fashion and progesterone directly inhibits pituitary LH secretion in estradiol-primed ewes. In the intact ewe, inhibition of LH secretion at the hypothalamic and pituitary levels is probably responsible for fluctuations in tonic LH secretion.

Progesterone inhibits pituitary LH secretion without inhibiting pituitary LH synthesis. In these studies progesterone selectively inhibits secretion of LH while synthesis of LH and the intrapituitary pool of LH remain unaffected. In the intact ewe, this mechanism may ensure that the necessary quantities of LH are present in the pituitary for a LH surge to occur each cycle.

Progesterone may be dependent on estradiol because estradiol enhances binding of progesterone by the pituitary. In ewes not treated with estradiol there is little progesterone binding in the pituitary, thus progesterone is unable to inhibit LH secretion. Estradiol enhances progesterone binding allowing progesterone to further enhance
its own binding and inhibit pituitary LH secretion. This mechanism likely occurs in the hypothalamus and pituitary and explains why both progesterone and estradiol are required to mimic tonic LH secretion in ovariectomized ewes.

Estradiol may have stimulatory effects on LH secretion, but these are dose and time dependent. In these studies a high dose of estradiol for a short time stimulated pituitary LH secretion while a high dose for a long time or a low dose for a short time had no effect on LH secretion. These different actions of estradiol on the pituitary begin to explain estradiol's positive and negative feedback effects on LH secretion during the estrous cycle of an intact ewe.

This study provides evidence that progesterone may directly inhibit LH secretion but other factors may change this action in a normal cycling ewe. Changes in GnRH pulse frequency may alter progesterone action and ovarian proteins may also alter progesterone action in the pituitary in a cycling ewe.
APPENDIX 1

METHODS AND MATERIALS

TRANSFORMATION

Plasmids containing cDNA inserts for either $\alpha$-subunit or LH-$\beta$ subunit were kindly provided by Dr. Nilson, Case Western Reserve University. The plasmid containing a cDNA insert for growth hormone was provided by Dr. Rottman, Case Western Reserve University. These plasmids were transformed into HB101 cells using the Hanahan procedure [Hanahan D, 1983]. Briefly, bacterial cells were treated with ice-cold solutions of calcium chloride and transfected with the forementioned plasmids. Transfected cells were then grown on agar with tetracycline and recombinant clones were selected for their tetracycline resistance. The identity of these clones was confirmed by restriction analysis using PstI to digest plasmids into fragments and comparing fragment sizes to a standard which was composed of pBR322 digested with HinFl.

1) 10 ugs plasmid DNA in dry form was obtained from John Nilson, resuspended in water to a concentration of 1 ug/ml.

2) HB101 cells were streaked onto a TB plate and incubated overnight at 37°C. The next morning one colony was dispersed into 15 mls of SOB broth and incubated at 37°C, 275 rpm to a density of 5 x 10^7 cells/ml ($O_{550}=0.5$).

3) Cells were transferred to 50 ml culture tubes and incubated on ice for 15 min, centrifuged at 500xg, 4°C, 15 min and the cells were resuspended in 5 ml of TFB and incubated at 4°C for 15 min.

4) Cells were collected again by the same centrifugation step and resuspended in 1.2 ml of TFB. 0.042 ml of DMSO was added with swirling and cells were incubated on ice for 5 min.

5) 0.042 ml of DTT was added with swirling and cells were incubated on ice for 10 min.

6) 0.042 of DMSO was added with swirling and cells were incubated for 5 min on ice.

7) Cells were dispersed into chilled polypropylene tubes (17x100 mm) and 10 ng (10 ul) of plasmid DNA from step 1 was added to cells with swirling and incubated on ice for 30 min.
8) Cells were incubated at 42°C for 90 sec then placed back on ice for 2 min.

9) 0.8 ml of SOB-glucose was added and cells were incubated at 37°C, 225 rpm for 1.5 hrs.

10) Cell suspension was plated onto TB+tet plates overnight.

11) Tetracycline resistant colonies were picked from the plates the following morning and dispersed into 6 mls of TB+tet broth and incubated overnight at 37°C, 225 rpm.

12) 2 mls of the cells were aliquoted out for rapid restriction analysis and 4 mls of glycerol were added to the remaining cells which were stored at -20°C for future use.

Buffers:
All chemicals are from Sigma Chemical Co., St. Louis, MO unless otherwise indicated.

TFB-Transformation Buffer: 10 mM 2-N-morpholinoethane sulfonic acid (adjusted to pH 6.2 using Potassium Hydroxide), 100 mM Rubidium Chloride, 45 mM Manganese Chloride·4 H₂O, 10 mM Calcium Chloride·2 H₂O, 3 mM Hexamine Cobalt (III) Chloride (Aldrich Chemical Co., Milwaukee, WI).

SOB-SOB medium: 2% (w/v) Bacto-Tryptone (Difco Laboratories, Detroit, MI), 0.5% Bacto-yeast extract (Difco Laboratories, Detroit MI), 10 mM Sodium Chloride, 2.5 mM Potassium Chloride, 10 mM Magnesium Chloride, 10 mM Magnesium Sulfate. SOB medium was prepared without Mg²⁺ and autoclaved. A 2 M stock of Mg²⁺ was used to make the medium 20 mM Mg²⁺. The final pH was 6.8 to 7.0.

TB+tet Broth-Terrific Broth plus tetracycline: One liter of TB was prepared by adding 100 ml of a sterile solution of 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ to a sterile solution containing 12 g Bacto-tryptone, 24 g Bacto-yeast extract, 4.0 ml glycerol and water to a final volume of 900 ml. Tetracycline was added to a final concentration of 15 ug/ml after sterilization.

TB+tet plates: To one liter of TB+tet broth add 15 g of Bacto-agar (Difco Laboratories, Detroit, MI). Following sterilization, add tetracycline and pour into 100x15 mm plates and let harden before use.
RAPID RESTRICTION ANALYSIS

1) Transfer 2 ml of cells from the transformation procedure to 2 ml eppendorf tubes and centrifuge at 500xg for 1 min and remove supernatant.

2) Resuspend the pellet in 100 ul of Sol. 1 and incubate on ice for 15 min.

3) Add 200 ul of Sol. 2, mix by inversion and incubate on ice for 5 min.

4) Add 150 ul of Sol. 3, mix by inversion and incubate on ice for 30 min.

5) Centrifuge for 5 min and transfer the supernatant to another tube.

6) Phenol/chloroform extract once and chloroform extract once.

7) Add 1 ml cold ethanol and precipitate on ice for 30 min, centrifuge 5 min, remove supernatant, rinse pellet with 70% ethanol, centrifuge again, dry pellets under vacuum.

8) Resuspend pellet in 30 ul of water, use 10 ul per restriction digest.

Solutions
All chemicals are from Sigma Chemical Co., St. Louis, MO and the enzymes are from Promega Inc., Madison, WI unless otherwise indicated.

Solution 1: 2 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO), 50 mM glucose, 10 mM Ethylenediaminetetraacetic Acid (EDTA), 25 mM Tris-HCl (pH 8.0). Prepare fresh daily from crystalline lysozyme, keep on ice.

Solution 2: 0.2 N Sodium Hydroxide, 1% Sodium Lauryl Sulfate (SDS), keep at room temperature, stable for 1 week.

Solution 3: 3 M Sodium Acetate (pH 4.8).

RNAse Solution: 1 mg/ml RNAse in 5 mM Tris-HCl (pH 8.0), heated at 100°C for 10 min.
LARGE-SCALE PLASMID ISOLATION

1) Plate transformed HB101 cells onto TB plates plus appropriate antibiotic. Use stocks stored in glycerol (from the transformation) at -20°C. Incubate overnight at 37°C.

2) Pick one colony and disperse into 250 ml of TB broth with appropriate antibiotic and incubate overnight at 37°C, 225 rpm. Up to 1000 ml of cells can be prepared this way and plasmid DNA isolated.

3) Pellet bacteria, 500xg, 20 min, 4°C.

4) Resuspend in 10 ml of sol. 1, stand on ice 10 min.

5) Add 20 ml of sol. 2, stand on ice 10 min.

6) Add 15 ml of sol. 3, stand on ice 30 min.

7) Centrifuge at 30,000xg, 45 min, recover supernatant.

8) Add 0.6 volume of r.t. isopropanol, mix, incubate r.t., 20 min.

9) Centrifuge 12,000xg for 30 min., r.t.

10) Wash with cold 70% ethanol, then dry pellet.

11) Resuspend plasmid DNA in 2 ml TE.

12) Add 100 ug/ml RNase A, incubate 1 hr, r.t.

13) Run plasmid DNA preparation through 1.5x30 cm Sepharose CL-4B column with TNE. Collect 25 1 ml fractions and check OD_{260/280}. The first peak (fractions approximately 19 - 22) contains the purified plasmid DNA. Ethanol precipitate, wash and dry. Resuspend in sterile water.

LABELING cDNA WITH [$^{32}$P]dCTP

To radiolabel cDNA, plasmid DNA was isolated using the alkali lysis method (Maniatis et al., 1989) and further purified by gel filtration. Plasmid DNA was digested with PstI and the cDNA inserts isolated by electroelution [Maniatis et al., 1982]. The isolated cDNA was radiolabeled to a specific activity of 1 x $10^9$ dpm/ug using ($\alpha$-$^{32}$P)dCTP and an oligolabeling kit (Pharmacia, Piscataway, N.J.).
NOTE: WEAR GLOVES and work behind beta-shield on disposable underpads when working with $^{32}$P.

1. Thaw $^{32}$P at room temp behind beta-shield. Warm Beta-Block in $37^\circ$ C incubator.

2. Calculate volume of cDNA insert to contain 50 ng DNA. Pipet DNA into microcentrifuge tube and add TE buffer (or dd H$_2$O) to 34 ul final volume.

3. Denature cDNA by placing tube in boiling water for 3 min. Remove tube from heat and IMMEDIATELY place on ice for at least 2 min.

4. Remove Reagent Mix and Klenow Fragment from Oligolabelling Kit from $-20^\circ$ C freezer. Keep on ice.

5. Set up work area with everything you'll need for the labeling process. CAUTION: Wear doubled gloves and work behind beta-blocking shield:

   To tube containing 34 ul denatured cDNA, add:
   1 ul Klenow Fragment
   10 ul Reagent Mix
   5 ul [32P]dCTP

   Vortex and centrifuge briefly to bring solutions together.

6. Incubate in Beta-Block at $37^\circ$ C for 60 min (or longer).

7) Purify reaction using Elutip-D mini-columns (Schleicher and Schuell (Dassel, West Germany)).

8) Pipet 1 ul of reaction mixture onto glass filter. When dry, place in scintillation vial and add 10 ml scintillation cocktail to determine total counts.

9) Pipet 25 ul Carrier DNA (Oligolabelling Kit) into a labeled microcentrifuge tube and add 1 ul of reaction mixture and vortex briefly. Add 1 ml cold ($4^\circ$ C) 10% TCA and vortex again. Incubate on ice for 15 min.

   Pour entire contents of tube onto glass-fiber filter in vacuum-filtering apparatus and rinse tube 2X with cold 10% TCA, followed by 2 rinses with 70% ethanol.

   When filter is dry, place in scintillation vial and add 10 ml cocktail to determine incorporated counts. Count CPM in total and incorporated samples with a scintillation counter (Program A13).
10. Calculate percent incorporated and CPM/ul (CPM Incorporated/5 ul x 100 dilution factor).

11. Determine the volume of purified, radiolabeled cDNA (use CPM Incorporated) needed to hybridize:
   (Optimal = 10-20 x 10^6 CPM/100 cm^2 blot area)

   \[
   \frac{15 \times 10^6 \text{ Incorporated CPM}}{100 \text{ cm}^2 \text{ blot area}} = \frac{\# \text{ CPM needed for blot}}{\text{blot size (cm}^2)\text{)}
   \]

12. Calculate volume (ul) to contain 15 x 10^6 CPM. Proceed to prehybridization and hybridization.

**Buffers:**

**TE Buffer (Tris EDTA) (pH 8.0)***

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>0.303 g</td>
</tr>
<tr>
<td>EDTA (0.5 M Stock)</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Q.S. to 250 ml with dd H_2O. Store at 4° C.

**10 mM EDTA (pH 8.0)**

Dilute 500 mM (pH 8.0) Stock EDTA solution 1:50 with dd H_2O.

**10% TCA (Trichloroacetic Acid)**

Dilute 100% TCA stock 1:10 with dd H_2O.

NOTE: (TCA is very hygroscopic. The addition of 1 ml dd H_2O/gram TCA (100% final conc.) when the bottle is first opened will reduce weighing errors).

**PLASMID DNA-DIGESTION AND cDNA ISOLATION**

1) Deliver 4 ug plasmid DNA (from large-scale plasmid isolation) into each microcentrifuge tube. (Include DNA from plasmid PBR322 cut with enzyme HinF-1 to give known size markers). Adjust volumes of dd H_2O and 10X buffer so that final concentration of buffer = 1X and final volume (with enzyme(s)) is 20 ul. Centrifuge briefly to bring all solutions together. Lastly, add 1 ul of appropriate restriction enzyme and recentrifuge.

Example: 1 ul DNA (P-NPY plasmid)

15 ul dd H_2O
2 ul 10X buffer (H)
1 ul PST1
1 ul EcoR1

NOTE: Keep restriction enzymes on ice.

Mix by flicking tube, recentrifuge, and incubate at 37° C for at least one hour (or overnight).
2) Prepare 4% polyacrylamide gel while DNA is incubating:

3) To each 20 ul of digested DNA, add 5 ul Loading Buffer. Mix by flicking tube and centrifuge to bring solutions together.
Load DNA into lanes (approx. 12.5 ul/lane). Run at 100 V for 2 hours until purple (farthest) dye has moved 2/3-3/4 of the way down the gel.
Remove gel to glass baking dish. Stain for 15-20 min with Ethidium Bromide prepared by adding 5 ul of 10 mg/ml stock to each 100 ml dd H_2O. Destain by rinsing 2X with dd H_2O.

4) Visualize with ultraviolet light. With razor blade, cut bands in gel corresponding to appropriate size of DNA.

5) Place gel strip into dialysis bag containing electrobuffer and immerse in horizontal gel chamber filled with electrobuffer. Pass 100 V of electrical current for 2 hr and reverse the polarity the last 2 minutes to release cDNA from the dialysis wall. Open the dialysis bag and carefully recover the cDNA solution.

6) Precipitate recovered cDNA by adding 0.1 volume NaAcetate and 2.5 volumes absolute (100%) ethanol. Incubate 30 min on ice or overnight at -20° C.

7) Centrifuge precipitate at 20,000 x g and wash pellet with 1 ml 70% ethanol. Recentrifuge.

8) Dry pellet in Speed Vac. Resuspend in small volume of water or TE buffer (50 ul). To maximize removal of DNA solution from Corex tube, remove as much as possible with a pipet and centrifuge tube briefly (3-5 min) at 2500-3000 RPM. Quantitate with "spot test" procedure.

Buffers:

Electrobuffer: 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 5 mM Sodium Chloride.

DNA Loading Buffer: 50% Glycerol, .25% Bromophenol Blue, 0.25% Xylene Cyanol FF.

SPOT TEST PROCEDURE

The final concentration of cDNA was determined using a "spot test" procedure [Greene and Guarente, 1987]. Briefly 1 ul of cDNA is mixed with 1 ul of 0.5% ethidium bromide and a standard curve with known amounts of DNA is photographed.
under UV light. The intensity of unknown is compared to the standard curve to determine the concentration of the sample.

1) Prepare standard line using DNA quantitated spectrophotometrically at 260 nm. In test tubes, prepare 50-100 µl of each DNA dilution: highest concentration of 20-25 ug/ml, dilute serially (2-fold) in TE buffer to lowest concentration of 0.25-0.5 ug/ml. Include TE buffer control.

2) Prepare 2-3 serial dilutions of unknown (DNA insert). Also test insert undiluted.

3) Pipet 2 ul of each standard and unknown onto a sheet of parafilm placed on UV light source (turned off). Add 2 ul 5 ug/ml ethidium bromide to each drop.

4) Visualize by UV illumination. Photograph to better quantify if samples are difficult to see. Quantitate by estimation. Multiply ug/ml by volume used to reconstitute insert (µg).

HYBRIDIZATION OF RNA BLOTS WITH cDNA PROBES

Prehybridization was carried out overnight at 42°C in 8 ml hybridization buffer/100 cm² membrane (hybridization buffer: 5x Denhardt's, 5x SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 50% deionized formamide, and 0.25% dried milk). Hybridizations were performed for 24 hours at 42°C in 4 ml hybridization buffer/100 cm² membrane with 2 x 10⁷ dpm of labeled cDNA (10⁹ dpm/µg) per 100 cm² membrane. After hybridization the nylon membranes were washed successively in 2x SSC, 0.1% SDS, 55°C. Kodak XAR-5 x-ray film and Dupont Cronex Intensifying Screens were used for autoradiography. Autoradiographic development times for α-subunit and LH-β subunit mRNAs were 4 and 5 days, respectively. After probing the total RNA for either α-subunit or LH-β subunit mRNA levels the nylon membranes were reprobed using the same prehybridization and hybridization conditions and ³²P-labeled GH cDNA. After hybridization the membranes were washed as just described and developed by autoradiography for twelve hours. The relative density of bands of specific (³²P)cDNA:mRNA heteroduplexes was measured using a Hoefer GS 300 scanning densitometer linked to a Perkin-Elmer LCI-100 integrator. Two 0.25 mm paths were scanned through each band on the autoradiograms and these values were averaged to give the density of each band. GH mRNA was unaffected by steroid treatment (n=6 ewes/treatment; p=0.69). Levels of α-subunit and LH-β subunit mRNA were normalized to a constant level of
GH mRNA and differences in LH subunit mRNA concentrations between treatments were compared using one-way ANOVA.

1) **Prehybridization**

To reduce non-specific binding (background), prehybridize blot with the following solutions for 1-6 hours (overnight incubation with formamide will require that you change the plastic bag before hybridization to assure that the corrosive action of formamide has not caused small leaks):

**For Biodyne membrane, prepare:**

<table>
<thead>
<tr>
<th>Prehybridization Solution, Concentration</th>
<th>(10 ml)</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ml 5 X SSC (20 X Stock)</td>
<td>2.5 ml 5 X</td>
<td></td>
</tr>
<tr>
<td>Sodium Phosphate (1 M Stock)</td>
<td>0.5 ml 0.5 M</td>
<td></td>
</tr>
<tr>
<td>SDS (20 % Stock)</td>
<td>0.05 ml 0.1 %</td>
<td></td>
</tr>
<tr>
<td>Powdered Milk</td>
<td>0.03 g 0.3 %</td>
<td></td>
</tr>
<tr>
<td>Deionized Formamide</td>
<td>5.0 ml 50 %</td>
<td></td>
</tr>
<tr>
<td>ddH₂O to 10 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**For Nitrocellulose, prepare:**

<table>
<thead>
<tr>
<th>Prehybridization Solution, Concentration</th>
<th>(10 ml)</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denhardt's (50 X Stock)</td>
<td>1.0 ml 5 X</td>
<td></td>
</tr>
<tr>
<td>SSPE (20 X Stock)</td>
<td>2.5 ml 5 X</td>
<td></td>
</tr>
<tr>
<td>SDS (20 % Stock)</td>
<td>0.05 ml 0.1 %</td>
<td></td>
</tr>
<tr>
<td>Powdered Milk</td>
<td>0.025 g 0.25 %</td>
<td></td>
</tr>
<tr>
<td>Deionized Formamide</td>
<td>5.0 ml 50 %</td>
<td></td>
</tr>
<tr>
<td>ddH₂O to 10 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calculate optimal volume of prehybridization solution:

\[
\text{8.0 ml Prehybrid. Solution} = \text{Amt. you need} \frac{100 \text{ cm}^2 \text{ of blot area}}{\text{your blot size}}
\]

2) Place dry blot in a Seal-a-Meal bag and seal all sides. Three sides should be sealed close to the blot and one side 1-2 inches away. Cut corner open to add solutions, push out all air bubbles and reseal bag.

3) Submerge in water bath and incubate at 37° C (non-stringent) or 40° C (stringent) for 1-6 hours (maximum 24 hours).

4) **Hybridization**

WEAR GLOVES when working with $^{32}$P.
For hybridization, prepare solutions as described above for Prehybridization for either membrane EXCEPT reduce Denhardt's to 2X (from 5X) to aid cDNA/RNA hybridization.

**Hybridization Solution (10 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denhardt's (50 X Stock)</td>
<td>0.4 ml 2 X</td>
</tr>
</tbody>
</table>

Calculate optimal volume of hybridization solution:

\[
\text{4.0 ml Hybrid. Solution} = \frac{\text{Amt. you need}}{100 \text{ cm}^2 \text{ of blot area}} \times \text{your blot size}
\]

5) At end of Prehybridization incubation, cut Seal-a-Meal bag open and push out all solution.

6) Refill with hybridization solution and push out as many bubbles as possible.

7) Determine volume of \(^{32}\text{P}\) to yield 10-20 cpm/100 cm\(^2\) blot area.

8) Boil radiolabeled cDNA in capped Eppendorf tube for 10 min. Immediately chill in ice for 2-3 min.

9) Pipet entire volume into Seal-a-Meal bag, push out remaining bubbles and seal. Mix solutions in bag by rubbing across blot several times.

10) Submerge in water bath and incubate overnight (12-20 hrs) at 37° C (lower stringency) or 42° C (higher stringency).

11) At end of incubation, wash blot:

12) Prepare 1500 ml Washing Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 X SSC</td>
<td>150 ml 2 X</td>
</tr>
<tr>
<td>20 % SDS</td>
<td>7.5 ml 0.1 %</td>
</tr>
</tbody>
</table>

and 500 ml Washing Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 X SSC</td>
<td>2.5 ml 0.1 X</td>
</tr>
<tr>
<td>20 % SDS</td>
<td>2.5 ml 0.1 %</td>
</tr>
</tbody>
</table>

WEAR GLOVES. Cut Seal-a-Meal bag open and press out radioactive solution into absorbent paper. Remove blot from bag and transfer to plastic container with lid.

13) Rinse quickly with 50 ml Solution A to remove hybridization solution.
NOTE: Discard all wash solutions into Radioactive Waste.

14) Add 500 ml Solution A and agitate vigorously for 5 min at room temp.

15) Discard into Radioactive Waste and refill with 500 ml Solution A.
16) Wash again. (Total 3 "higher salt" washes.)

17) Discard wash solution into Radioactive Waste and seal blot into Seal-a-Meal bag.

18) Place one or two spots of fluorescent nail polish on one corner of the bag and let dry. (This will help to orient the location of the blot on the autoradiograph.)

IN DARKROOM, sandwich X-ray film between blot and amplifying screen.

Expose film at -80°C between two thick (Sigma) catalogues.

Buffers:

**20 X SSC**  
Final Concent. (20 X)  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>175.3 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>88.2 g</td>
</tr>
</tbody>
</table>

Q.S. to 1000 ml with dd H₂O. Adjust pH to 7.0. Aliquot into 200-250 ml bottles and sterilize by autoclaving. Store at 4°C.

**1 M Sodium Phosphate** (for Bicodyne only)  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>5.562 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>7.794 g</td>
</tr>
</tbody>
</table>

Q.S. to 100 ml with dd H₂O. Adjust pH to 6.5. Store at 4°C.

**20 X SSPE** (for Nitrocellulose only)  
Final Concent. (20 X)  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>174 g</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>27.6 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>7.4 g</td>
</tr>
</tbody>
</table>

Q.S. to 1000 ml with dd H₂O. Adjust pH to 7.0. Aliquot into 200-250 ml bottles and sterilize by autoclaving. Store at 4°C.

**50 X Denhardt's**  

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>
(BSA, Pentax Fraction V)  
Q.S. to 500 ml with dd H₂O. Aliquot into 10-15 ml tubes covered with foil to exclude light. Store at -20°C.

**SDS (20%)**  
Sodium lauryl sulfate 20 g  
Sprinkle SDS into beaker containing approx. 80 ml dd H₂O. Mix and heat to dissolve. Q.S. to 100 ml with dd H₂O. Autoclave to sterilize.  
NOTE: Freezes at 4°C. Bring to room temp to reliquefy.

**Deionized Formamide**  
Add 5 grams Mixed Bed Resin per 100 ml formamide. Cover beaker with foil to exclude light and mix for 30 min at room temp. Filter 2X through Whatman #1. Store at -20°C in 10-15 ml aliquots in tubes wrapped in foil.

**SLOT-BLOT PREPARATION**

The relative amounts of mRNA for LH-β subunit, LH-α subunit and GH were measured using the Fast Blot technique [Costanzi and Gillespie, 1987]. Pituitary RNA (0.5 µg/slot) was immobilized to Biotrans nylon membrane (ICN Biomedicals, Irvine, CA) using a slot blot apparatus (BRL Inc., Gaithersburg, MD) and baked at 80°C for 1 hour. This concentration of total pituitary RNA (0.5 µg) was used because this concentration lies within the range of total RNA concentrations (0.1-1.5 µg total RNA) that produced a linear increase in the hybridization signal intensity when probed with ³²P-labeled cDNA and visualized by autoradiography.  
1. Cut membrane to size appropriate for slot blot apparatus. Notch upper left corner to mark position A-1. Save protective papers to measure area for hybridization.

2. Wet membrane with dd H₂O and then soak in 20X SSC for 1 hr at room temp. Meanwhile clean manifold with 1 M NaOH or Alconox and rinse well with dd H₂O.

3. Load wet membrane into manifold. Smooth away all bubbles. Assemble manifold with trough on the bottom and slots together (upper layers will create a shared groove when assembled correctly). Clamp manifold together and tighten all screws simultaneously or alternating corners to prevent warpage.

4. Fill all slots with 10X SSC and apply suction until all fluid is passed through the membrane. Turn off vacuum and refill.  
(NOTE: Do not let nitrocellulose dry out.)
Prepare RNA Samples:
1. Determine the concentration of RNA/ml by absorbance at 260 nm. Calculate volume to contain appropriate final concentration (20 ug RNA maximum) plus some additional (0.5-1 ug) for pipetting loss. If greater than 10 ul is needed, adjust volumes for Denaturing Solution and 20X SSC for loading.

2. Prepare denaturing solution first, then add RNA to it.

NOTE: Use sterile pipet tips and gloves when working with RNA.

Denaturing Solution
3. Pipet into Eppendorf tube (one tube per sample):

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Formamide</td>
<td>20 ul</td>
</tr>
<tr>
<td>Formaldehyde (37% Stock)*</td>
<td>7 ul</td>
</tr>
<tr>
<td>20X SSC</td>
<td>2 ul</td>
</tr>
<tr>
<td>dd H₂O to 39 ul final volume with RNA (H₂O + RNA = 10 ul)</td>
<td>1 X</td>
</tr>
</tbody>
</table>

4. Mix solutions by vortexing.

5. Pipet RNA into above solution and mix immediately by vortexing.

6. Incubate for 15 min at 68° C. Cool immediately on ice.

*CAUTION: Formaldehyde is toxic. Use in fume hood and wear gloves. Check pH of stock formaldehyde, it should be greater that 4.0.

7. Add 2 volumes 20X SSC to denatured RNA sample.

8. Calculate RNA concentration in final solution and pipet appropriate volume into Slot Blot well. Apply gentle suction to draw sample through membrane. Rinse slots 2 times by filling wells with 10X SSC. (NOTE: If volume is less than 100 ul, pipet some 10X SSC into slot first, then add RNA solution.)

9. Remove membrane from manifold and allow it to dry completely at room temp between to pieces Whatman #1 filter paper and bake for 2 hours at 80° C in a vacuum oven. Store baked membrane at room temp.

Buffers:
20 X SSC

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>175.3 g</td>
</tr>
<tr>
<td></td>
<td>3.0 M</td>
</tr>
</tbody>
</table>
Sodium citrate 88.2 g 0.3 M
Q.S. to 1000 ml with dd H₂O. Adjust pH to 7.0. Aliquot into 200-250 ml bottles and sterilize by autoclaving. Store at 4 °C.

GUANIDINE ISOTHIOCYANATE ISOLATION OF RNA

Total cellular RNA was extracted from the harvested pituitaries by homogenization in 4M guanidine isothiocyanate with a Brinkman homogenizer (Brinkman Instrument Co., Westbury, NY) at high speed for 45 seconds and centrifuged at 10,000xg for 30 minutes. The supernatant was layered over a 4 ml, 5.7M cesium chloride cushion and centrifuged overnight at 100,000xg [MacDonald et al., 1987]. The supernatant was removed and the RNA pellet was resuspended in sterile water and concentrations determined by measuring absorbance at 260 and 280 nM. Isolated RNA was stored at -70°C until further use.

NOTE: Ribonucleases are everywhere! Guanidine isothiocyanate inactivates RNases, but in all steps before and after those where the sample is mixed with GI, degradation of RNA may occur. Wear latex examination gloves and change them frequently. Handling of tubes that contain RNA should be done with gloves.

DAY 1
Autoclave for 30 min all solutions except Guanidine isothiocyanate. In addition, autoclave 50 ml dd H₂O, 5 inch pasteur pipets, tips for pipettors, 1 sterile 5 ml pipet, Polyallomer ultracentrifuge tubes (14 x 89 mm) and microfuge tubes (Brinkman, conical bottom with snap-cap).

DAY 2
Sign up for overnight use of the ultracentrifuge.

Wear gloves. Remove tissues from -70°C freezer into liquid nitrogen. Break off thumb-sized piece of frozen tissue (approx. 2 grams) with hammer. Place tissue in mortar containing liquid nitrogen and pulverize by pounding and grinding with pestle until tissue is a fine powder. Add liquid nitrogen often. KEEP TISSUE FROZEN!

Weigh tissue sample by first labeling and weighing 50 ml Nalgene test tubes, then chilling by placing in liquid nitrogen. Add slurry of ground tissue in liquid nitrogen to test tube as it sits on the balance. As liquid nitrogen evaporates, take a weight measurement of the tube. (Approx. 1-
1.5 grams). Return tubes immediately to liquid nitrogen. KEEP TISSUE FROZEN!

Add 7 ml Guanidine isothiocyanate solution and 70 ul beta mercaptoethanol (1.0% final concentration) to each 1 gram tissue (approx.). When tissue has warmed slightly, but not thawed, homogenize with Polytron at lower setting (3 or 4), then blend at setting 6 or 7 for 20 seconds.

Add sarkosyl to 0.5% (177 ul/7 ml GI) and mix well by vortexing.

Transfer to 15 ml Corex centrifuge tubes. Balance opposite tubes to 0.01 grams with GI.

Centrifuge in J2-21 using JS 13.1 rotor at 8,000 rpm (10,000 x g) for 20 min at 20° C. Supernate should be clear but may be colored. If cloudy, centrifuge again for a longer time. (Supernate will contain RNA).

Overlay supernate onto 4 ml cesium chloride solution in an autoclaved polyallomer ultracentrifuge tube.

Prepare ultracentrifuge:
Use matched carrier pairs for SW 40 rotor. Slide tubes into carriers and add GI solution till nearly full. Weigh opposite tubes and caps, and balance with GI solution as needed (0.01 gram tolerance). Cap tightly, use screw driver to tighten (in drawer at northwest end of bench).

Ultracentrifuge for 20-24 hours at 32,000 rpm at 20° C. Set at 4 acceleration (2 min to reach 170 rpm), 0 deceleration (no brake).

Monitor centrifuge until maximum speed has been reached (4-5 min).

DAY 3
WEAR GLOVES. Remove tubes from ultracentrifuge. (Wash carriers and caps with warm water if necessary, rinse with dd H2O).

Proteins and lipids will be near the top, DNA will appear in a whitish band near bottom third of the GI layer, and RNA will be a small pellet on the bottom of the tube (however, RNA may not be visible until cesium chloride is removed).

Using an autoclaved pasteur pipet, remove supernate carefully and slowly starting with tip placed at the top of the GI layer, working downward as solution level drops. (NOTE: Guanidine is a powerful denaturant; dispose in hazardous waste container).

Rub sides of tube with pipet to remove proteins and lipids. Remove DNA layer to the bottom of the lower white band
(approx. 1.5 cm from the bottom) (if DNA layer is large, pierce with pasteur pipet) and thoroughly wash down the sides with fresh GI solution.

The removal of the GI layer and the washing of the insides of the tube may need to be repeated.

Finally, with a new pasteur pipet (autoclaved) remove last layer of cesium chloride. Invert the tube and using a razor blade, cut tube 1-1.5 cm above the bottom and drain excess solution on a Kimwipe.

Resuspend RNA pellet in 100-200 ul SET buffer with sterile pipet tip; mix well by drawing up and down in tip. Transfer to sterile Brinkman microcentrifuge tube and precipitate RNA with 0.1 volume 3 M Na Acetate (pH 4.5-5.0) and 2.5 volumes absolute ethanol (100%). Freeze at -20° C overnight.

**DAY 4**
Centrifuge precipitated RNA in microcentrifuge for 10 min, wash pellet in 1 ml 70% ethanol (prepared with autoclaved dd H2O), and recentrifuge. Decant supernate, but save in beaker to see if any RNA pellet was lost.

Dry sample in Speed-Vac for 10 min. until very dry (repeat if necessary). Start centrifuge, then turn on pump and close vacuum line to evacuate centrifuge chamber. Reverse steps to remove samples.

Dissolve pellet in 50-200 ul sterile water (depending on size of pellet). Record volume.

Measure amount of RNA spectrophotometrically by adding a small sample (5 - 10 ul) of RNA solution to microcuvette containing 900 ul non-sterile dd H2O (dilution = 1:181 or 1:91, respectively). Fill a second cuvette with 900 ul dd H2O to blank between wavelength changes. Read absorbance at 260 and 280 nm. Ratio of absorbance values at 260/280 should be between 1.8 and 2.0.
One A260 equals 40 RNA ug/ml. (Yield from calf muscle is approx. 200 ug/gram tissue). Store at -70° C.

**Buffers:**

**Guanidinium Isothiocyanate (4 M)**
guanidine isothiocyanate (FW 118.16) 50 g
1 M Tris-base, pH 7.5 (FW 121.1) 10 ml
Q.S. to 100 ml with dd H₂O. Filter particulates with Whatman 1MM paper. Solution is stable indefinitely.

**Sodium Acetate** (3 M) (pH 4.5 - 5.0)

Na acetate 3H₂O (FW 82.03) 12.3045 g

Dissolve in 15-20 ml dd H₂O. Adjust pH with glacial acetic acid. Q.S. to 50 ml with dd H₂O. Autoclave for 30 min.

**EDTA** (200 mM) (pH 7.0)

EDTA disodium salt (FW 372.24) 37.224 g

Dissolve in 50-100 ml 5 N NaOH. Add 50-100 ml dd H₂O. Adjust pH. Q.S. to 500 ml with dd H₂O. Autoclave for 30 min.

**SDS** (20%)

Sodium lauryl sulfate (FW 288.4) 20 g

Sprinkle SDS into beaker containing approx. 80 ml dd H₂O. Mix and heat to dissolve. Q.S. to 100 ml with dd H₂O. Autoclave for 30 min.

NOTE: Freezes at 4° C. Bring to room temp. to reliquefy.

**Cesium Chloride** (5.7 M)

cesium chloride (FW 168.36) 48 g

200 mM EDTA stock (sterile) 25 ml

Q.S. to 50 ml with dd H₂O. Millipore filter (0.45 uM) and autoclave for 30 min.

**SET Buffer**

Final Conc.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-base, pH 7.4</td>
<td>1.0 ml 10 mM</td>
</tr>
<tr>
<td>200 mM EDTA stock</td>
<td>2.5 ml 5 mM</td>
</tr>
<tr>
<td>20% SDS stock</td>
<td>0.5 ml 0.1 %</td>
</tr>
</tbody>
</table>

Q.S. to 100 ml with dd H₂O. Millipore filter (0.45 uM) and autoclave for 30 min.

**Sarkosyl** (20%)

sarkosyl (FW 279.40) 20 g

Sprinkle into beaker containing approx. 80 ml dd H₂O. Mix and heat to dissolve. Q.S. to 100 ml with dd H₂O. Millipore filter (0.45 uM) and autoclave for 30 min.

FORMALDEHYDE GELS AND RNA TRANSFER

1) Prepare 1.5% agarose gel containing a final concentration of 2.2 M formaldehyde.

2) Ethanol precipitate and dry down aliquot of RNA sample. Resuspend in 15 ul denaturing solution, incubate on ice 1 hr.
3) Heat at 65°C for 15 min, chill on ice.

4) Add 5 μl of loading buffer, mix and load onto agarose gel. Circulate running buffer in apparatus from (+) to (-).

5) Carry out electrophoresis in a cold room at 30 V overnight.

6) Stain with 10 μg/ml acridine orange in 10 mM phosphate buffer (pH 6.5). Visualize RNA under ultraviolet light.

Transfer RNA to nylon membrane

1) Saturate Whatman 3MM paper in 20X SSC, place over glass platform so ends are in reservoir. Add 20X SSC to reservoir.

2) Invert and place the gel on the 3MM paper. Remove all air bubbles.

3) Surround the gel with strips of film.

4) Place biodyne membrane onto gel after prewetting the membrane in water and 20X SSC.

5) Place 2 sheets of 3MM paper over membrane, then a 3 inch layer of paper towel, a 500 g weight, and plastic. Transfer overnight.

6) Remove the membrane, air dry and bake at 80°C for 1 hr. Store in plastic at r.t.

Buffers:
20X SSC: 3 M Sodium Chloride, 0.3 M Sodium Citrate
Sterilize by autoclaving. Store at 4°C.

Running Buffer: 10 mM Sodium Phosphate Dibasic, 10 mM Sodium Phosphate Monobasic (pH 7.0).

10X Denaturing Buffer: 200 mM HEPES, 50 mM Sodium Acetate, 10 mM EDTA.

Denaturing Solution: 12% 10X Denaturing Buffer, 50% deionized formamide, 2.2 M formaldehyde (pH>5).

Loading Buffer:
Glycerol (50% final) 2.5 ml
EDTA (200 mM stock, 1 mM final) 0.030 ml
Bromphenol Blue (0.25% final) 0.013 g
Xylene Cyanole FF (0.25% final) 0.013 g
Q.S. to 5 ml with dd H₂O. Autoclave for 30 min.
LUTEINIZING HORMONE RADIOIMMUNOASSAY

Serum concentrations of LH were measured using a previously validated RIA [Niswender et al., 1969; Wise ME, 1990] with $^{125}$I-labeled NIADDK-ovine LH-I-3 as tracer. NIADDK-ovine LH-25 served as standard. The ovine LH antibody CSU-204 was kindly supplied by Dr. Niswender, Colorado State University. The lower limit of detection was 0.4 ng/ml as determined by inhibition of total binding to 90%. The intra- and interassay coefficients of variation were less than 9% and 15%, respectively.

Tissue content of LH was determined by homogenizing a small portion of the collected pituitary tissue and measuring the concentration of LH in this homogenate using the previously described radioimmunoassay for serum LH. The total protein content of the homogenate was determined by Bradford colorimetric protein assay [Bradford MM, 1976] and the pituitary content of LH expressed as ng LH/ug total pituitary protein.

1) Ovine LH was iodinated using the Chloramine-T method and purified by filtration through a Sephadex G25-150 column (0.7x23 cm).

2) Iodinated LH was diluted to 300,000 cpm/ml in 0.1% PBS-G.

3) LH standards were made in 0.1% PBS-G and ranged from 0.1 to 50 ng/ml.

4) 200 uls of serum or standard were added to 12x75 mm tubes containing 300 uls of 0.1% PBS-G.

5) 200 uls of first antibody was added, tubes vortexed and incubated overnight at 4°C.

6) 100 uls of diluted, iodinated LH was added, mixed and reaction incubated at 4°C overnight.

7) 200 uls of second antibody was added, mixed and reaction incubated for 48 hrs at 4°C.

8) 2 mls of PBS was added and tubes were centrifuged at 1000xg for 30 minutes. Supernatant was decanted and cpm,s were counted in the pellet with a gamma-counter. Concentrations of LH in serum were determined by extrapolation from the standard curve.

Buffers:
PBS-Phosphate-Buffered Saline: Dissolve 35.75 g Sodium Chloride in 200 ml deionized water. Add 0.44 g Merthiolate, 25 ml of 0.5 M Sodium Phosphate monobasic and 65 ml of 0.5 M Sodium Phosphate dibasic. Dilute to 4.375 liters and adjust pH to 7.0.

0.1% PBS-G: PBS plus 0.1% gelatin.

PBS-EDTA: PBS plus 18.6% EDTA (disodium salt) and 1% Merthiolate.

1:400 NRS in PBS-EDTA: Dilute normal rabbit serum 1:400 with PBS-EDTA.

First Antibody: Dilute CSU-204 antibody stock 1:25,000 with 1:400 NRS in PBS-EDTA.

Second Antibody: Dilute anti-sera ewe# 1931 (10/31/88) 1:20 with PBS-EDTA.

**ESTRADIOL RADIOIMMUNOASSAY**

Serum concentrations of estradiol were determined in a single assay using a modification of a previously described RIA [Wise, 1987]. Briefly, duplicate 1 ml aliquots of serum were extracted with methylene chloride and chromatographed through a 0.5 x 6.0 cm column containing LH-20 Sephadex (Sigma Chemical Co., St. Louis, MO), eluted with 9:1 benzene:methanol, dried and reconstituted in 0.01 M phosphate buffered saline-0.1% gelatin (pH 7.1). The tracer was [2,4,6,7, 17-3H] Estradiol (Amersham Co., Arlington Heights, IL) and the estradiol antibody CSU-244 was kindly provided by Dr. Niswender. 17β-Estradiol (Sigma Chemical Co., St Louis, MO) served as standard. The sensitivity of the assay was 2.5 pg/ml and the intra-assay coefficient of variation was 9.7%.

1) Serum was extracted with 10:1 methylene chloride:serum.

2) Extracted samples were resuspended in 9:1 benzene:methanol and chromatographed through a 0.5x6.0 cm Sephadex LH-20 column.

3) Fractions with chromatographed serum were dried, resuspended in 500 ul 0.1% PBS-G and transferred to 12x75 mm tubes.

4) Estradiol standards were made in 0.1% PBS-G and ranged from 2.5 to 100 pg/ml.
5) 200 ul of estradiol antibody was added to samples and standards and 0.1% PBS-G was added to bring volume to 900 ul. Reactions were vortexed and incubated overnight at 4°C.

6) 100 ul of tritiated estradiol was added, mixed and incubated overnight at 4°C.

7) 400 ul of 0.5% DCC was added, vortexed and incubated on ice for 10 min. Reactions were spun at 1000xg for 30 min, supernatants decanted and dpms counted on a beta counter. Concentrations in samples were determined from extrapolation from the standard curve.

Buffers
PBS-Phosphate-Buffered Saline: Dissolve 35.75 g Sodium Chloride in 200 ml deionized water. Add 0.44 g Merthiolate, 25 ml of 0.5 M Sodium Phosphate monobasic and 65 ml of 0.5 M Sodium Phosphate dibasic. Dilute to 4.375 liters and adjust pH to 7.0.

0.1% PBS-G: PBS plus 0.1% gelatin.

Estradiol Antibody: A 1:150,000 dilution of anti-sera from ewe# 244 with 0.1% PBS-G.

Tritiated Estradiol: \([2,4,6,7,17-^3\text{H}]\)Estradiol was dried and resuspended in 0.1% PBS-G to 100,000 dpm/ml.

0.5% DCC: 0.5% activated charcoal, 0.5% dextran made up in 0.1% PBS-G.

PROGESTERONE RADIOIMMUNOASSAY

Serum concentrations of progesterone were measured in one assay using a RIA kit (Diagnostic Products Co., Los Angeles, CA). The sensitivity was 1.0 ng/ml as determined by inhibition of total binding to 90% and the intra-assay coefficient of variation was 8.1%. Progesterone was determined using the coat-a-count kit (Diagnostics Products Co., Los Angeles, CA)

1) 100 ul of sample or kit standard was mixed with 1 ml of buffered \([^125\text{C}]\)Progesterone from the kit and incubated at r.t. for 3 hrs.

2) Decant thoroughly and count on a gamma counter. Concentrations in samples were determined from extrapolation from the standard curve.
A cytosolic progesterone receptor assay (Clark et al., 1987) was used for determination of progesterone binding capacity by saturation analysis. \([1,2,6,7-^3\text{H}]\)Progesterone (Amersham, Arlington Heights, IL) was used as the labeled ligand used and progesterone (Sigma, St. Louis, MO) was the unlabeled ligand. Pituitaries were removed, minced, weighed, and suspended in ice-cold TG buffer (10 mM Tris, 30% glycerol v/v, pH 7.4) with tissue/volume = 40 mg/ml. Within one hour the suspensions were homogenized with a Brinkman homogenizer (Brinkman Instruments Co., Westbury, NY) using two 5 second high speed bursts and centrifuged at 35,000xg for one hour. The cytosolic suspension was added to tubes containing 12 or 15 nM \(^3\text{H}\)-Progesterone plus or minus 100-fold excess of cold progesterone in a final volume of 0.5 ml and incubated at 30°C for 45 minutes. Following incubation 0.25 ml of ice-cold DCC solution (1.0% charcoal, 0.05% dextran in TG buffer) was added to the tubes with vortexing and immediately centrifuged at 2500xg for 6 minutes. The supernatant was poured off, 10 mls of aqueous counting scintillant was added and the assay was counted on a liquid scintillation counter (LKB Instrument Inc., Gaithersburg, MA). Saturation analyses of pituitaries from HPD ewes were done in triplicate per tissue at 12 and 15 nM labeled progesterone.

1) \([1,2,6,7-^3\text{H}]\)Progesterone was dried and resuspended in TG buffer at a stock concentration of 90 nM. A cold progesterone stock of 1 ug/ml was also made.

2) Pituitaries were removed, minced, weighed, and suspended in ice-cold TG buffer with tissue/volume ratio = 40mg/ml.

3) Within 1 hr the suspensions were homogenized with a Brinkman homogenizer (Brinkman Instruments Co., Westbury, NY) using two 5 second high speed bursts and centrifuged at 35,000xg for one hour.

4) The cytosolic suspension was added to tubes containing 12 or 15 nM \(^3\text{H}\)-Progesterone plus or minus 100-fold excess of cold progesterone in a final volume of 0.5 ml and incubated at 30°C for 45 minutes.

5) Following incubation 0.25 ml of ice-cold DCC solution (1.0% charcoal, 0.05% dextran in TG buffer) was added to the tubes with vortexing and immediately centrifuged at 2500xg for 6 minutes.
6) The supernatant was poured off, 10 mls of aqueous counting scintillant was added and the assay was counted on a liquid scintillation counter (LKB Instrument Inc., Gaithersburg, MA).

7) Dpms specifically bound were converted to fM bound/g tissue weight.
REFERENCES


Batra SK, Miller WL. Progesterone decreases the responsiveness of ovine pituitary cultures to luteinizing hormone-releasing hormone. Endocrinol 1985; 117:1436-1440.

Batra SK, Miller WL. Progesterone antagonizes the ability of porcine ovarian inhibin to sensitize ovine pituitary cell culture to luteinizing hormone-releasing hormone: Dependence on ovaries in vivo. Endocrinol 1986; 119:1933-1938.


Clarke IJ, Cummins JT. The temporal relationship between gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in ovariectomized ewes. Endocrinol 1982; 111:1737-1739.


Huang ESR, Miller WL. Effects of estradiol-17β on basal and luteinizing hormone releasing hormone-induced secretion of luteinizing hormone and follicle-stimulating hormone by ovine pituitary cell culture. Biol Reprod 1980; 23:124-134.


Karsch FJ, Foster DL, Bittman EL, Goodman RL. A role for estradiol in enhancing luteinizing hormone pulse frequency during the follicular phase of the estrous cycle of sheep. Endocrinol 1983; 113:1333-1339.


Ke FC, Ramirez VD. Binding of progesterone to nerve cell membranes of rat brain using progesterone conjugated to $^{125}$I-bovine serum albumin as a ligand. Neuroendocrinol 1990; 54:467-472.


Lumpkin MD, DePaolo LV, Negro-Villar A. Pulsatile release of follicle-stimulating hormone in ovariectomized rats is inhibited by porcine follicular fluid (inhibin). Endocrinol 1984; 114:201-206.


Mercer JE, Clements JA, Funder JW, Clarke IJ. Luteinizing hormone-β mRNA levels are regulated primarily by gonadotropin-releasing hormone and not by negative estrogen feedback on the pituitary. Neuroendocrinology 1988; 47:563-566.


