Luteinizing Hormone-Releasing Hormone Gene Expression in the Bovine Brain: Anatomical Localization and Regulation by Ovarian State

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INTRODUCTION

Luteinizing hormone-releasing hormone (LHRH) is a decapeptide synthesized by neurons in the rostral preoptic area of the bovine brain. It is then transported to the median eminence (infundibulum), where it is released into the hypothalamic-hypophyseal portal system and delivered to the pituitary. The pulsatile release of LH by the pituitary is tightly coupled to the pulsatile release of LHRH in pigs [1], sheep [2,3], rats [4], and monkeys [5].

Immunocytochemical analysis has revealed that the distribution of LHRH neurons forms a loosely arranged continuum in the diagonal band of Broca, organum vasculosum of the lamina terminalis (OVLT), medial preoptic area (POA), and the rostral-most anterior hypothalamus in the bovine brain [6,7]. A similar distribution of LHRH neurons was reported in the sheep [8,9]. In rats, expression of the LHRH gene fluctuates with the endocrine status of the animal. Therefore, we designed an experiment to examine cellular levels of LHRH and mRNA in the brains of cows representing two different endocrine states—one with (presumably) high LHRH secretion and one with low LHRH secretion.

MATERIALS AND METHODS

Animals

Ten crossbred heifers (18–26 mo of age) were divided into two groups. One group, designated OVX, consisted of five heifers ovarioctomized 14–16 wk before the study. The second group, designated LUTEAL, consisted of five heifers killed during the mid-luteal phase (Days 9–10, Day 0 = estrus) of the estrous cycle. Serum concentrations of progesterone were used to verify stage of the estrous cycle. All heifers were slaughtered in the abattoir of the Texas A&M University Rosenthal Meat Science and Technology Center. Slaughter procedures conformed to State and Federal guidelines for humane slaughter of animals. After stunning was performed by captive bolt, a tissue fragment was dissected from each brain. The anterior border of the frag-
In Situ Hybridization

Brains were sectioned (7 μm) on a cryostat and mounted onto organosilane-subbed slides. Sections were collected throughout the diagonal band of Broca, POA, and the rostral hypothalamic area. Sections were also collected from the mediobasal hypothalamus of two of the brains (one per group). Mounted brain sections were fixed in 4% paraformaldehyde in PBS (pH 7.4), immersed in triethanolamine, then dehydrated in ethanol. For in situ hybridization, we used a 59-mer deoxyoligonucleotide complementary to the region of the human LHRH mRNA coding for amino acids 5 to + 15 of prepro-LHRH [15]. This probe shows 90% homology to the rat LHRH gene [16] and is presumably similar to the bovine sequence. The oligomer was 5’ end-labeled with 32P using T4 polynucleotide kinase (Promega Corp., Madison, WI). The probe was dissolved in 1:1 dehydrated and diphenyl ether (Kodak D-19 and fixer) and lightly counterstained with cresyl violet.

Quantification of LHRH mRNA

All sections were carefully examined under a light microscope; and for each brain, the region showing maximal numbers of LHRH-expressing neurons was determined. Then, three brain sections (per animal) from this region, selected at 100-μm intervals, were further analyzed to determine: number of cells expressing the LHRH gene; number of grains per labeled cell; and total number of grains in all labeled cells. The number of labeled cells per section was determined by visual observation. The reduced silver grains, proportional to LHRH mRNA [17], were quantified in each labeled cell (in pixels) via a Bioquant System IV image analysis system. For all labeled cells, the number of pixels was at least five times background.

Analysis of LH and Progesterone Concentration

Serum concentration of LH was determined by the double-antibody radioimmunoassay (RIA) originally described by Niswender et al. [18] with the following modifications: 1) anti-ovine LH serum (TEA #35) at a final dilution of 1:500,000 in 0.1% egg white PBS, pH 7.0, was used as the first antibody; 2) bovine LH (USDA-bLH-I-2), radiiodinated (iodogen method) with 125I, was added on the second day; 3) sheep anti-rabbit gammaglobulin (P-4, Antibodies, Inc., Davis, CA), used at a dilution of 1:40 in 10% polyethylene glycol in PBS, was added on the third day; 4) 24 h after the addition of the second antibody, the assay tubes were centrifuged and supernatant was aspirated and precipitate counted with a Micromedic MACC system; and 5) all values were expressed in terms of the NIH-bLH-B-10 reference preparation. Sensitivity of the LH assay was 0.1 ng/tube. The intraassay coefficient of variation (CV) for standard serum from ovariectomized cows was 4.73%.

Serum progesterone concentrations were quantified by the radioimmunoassay described by Mosley et al. [19]. The first antibody was GDN-337 and was used at a final dilution of 1:1,750. The sensitivity of the assay and the intraassay coefficients of variation were 7.8 pg/tube and 4.4%, respectively.

Statistical Analyses

The Wilcoxin Rank Sum test was used to compare number of LHRH-expressing neurons between experimental groups as well as for comparing total numbers of pixels representing LHRH and mRNA. This test was also used to evaluate differences in serum concentrations of LH and progesterone between the groups. The Kolmogorov-Smirnov Two-Sample test was used to test for differences in distributions of the number of grains per cell between the groups [20].

RESULTS

LHRH mRNA-containing neurons were well labeled throughout the diagonal band of Broca, OVLT, and the rostral most anterior hypothalamic area. Figure 1 is a drawing of a representative tissue section showing the locations of labeled neurons. This tracing was made with the use of a Zeiss microscope with a camera lucida attachment. Typically, labeled neurons had greater than 1000 pixels covered by silver grains and were clearly distinguishable from unlabeled neurons which typically possessed fewer than 50 pixels. Figure 2 is a photomicrograph showing four LHRH-expressing neurons in the bovine POA as well as several unlabeled cells. The distribution of the labeled neurons was very similar to the immunocytochemical results described by Leshin et al. [6]. For all animals, the medial POA was the
brain region with the highest number of labeled cells, with fewer cells found in the more rostral and caudal areas. Additionally, some cells were found in the septum and the nucleus of the stria terminalis.

Serum Hormone Concentrations

Radioimmunoassay of serum samples for LH and progesterone confirmed expectations regarding the hormonal differences between the two experimental groups. The OVX
females had higher serum concentrations of LH than the LUTEAL group (2.61 ± 0.85 vs. 0.39 ± 0.05 ng/ml; p < 0.01). The LUTEAL group had higher serum progesterone concentrations than the OVX group (2.0 ± 0.37 vs. 0.08 ± 0.007 ng/ml; p < 0.01), verifying that each animal in the LUTEAL group was indeed in the mid-luteal phase of the estrous cycle.

**Number of Labeled LHRH Cells**

For each analyzed section (three per animal), the number of labeled cells was determined by visual observation using a light microscope. The analyzed brain sections from OVX cows averaged 12.6 ± 1.6 labeled LHRH neurons per section while the LUTEAL group brain sections averaged just 8.3 ± 1.1—a 34% decrease (Fig. 3, p < 0.05). It appears that an ovarian factor, in likelihood progesterone, suppresses some LHRH neurons to a point at which they either stop expressing the LHRH gene or express it only at undetectable levels.

**Amount of LHRH mRNA Per Cell**

For all labeled cells, the number of pixels per cell (proportional to reduced silver grains) was determined. This value is proportional to number of LHRH mRNA copies per cell [17]. The LHRH neurons from OVX animals had a mean of 3619 ± 280 pixels/cell with an even distribution above and below the mean. The LUTEAL animals had a mean of 2592 ± 379 pixels/cell, with a distribution significantly skewed to the left (p < 0.01) of the OVX group as represented in Figure 4. This shift indicates that LHRH neurons from the LUTEAL animals had fewer copies of LHRH mRNA per cell than the OVX animals.

**Total Number of Pixels**

The number of pixels in all labeled neurons was totalled to yield a measure of total LHRH mRNA in brain tissue samples from each animal. LUTEAL animals averaged 57% fewer (p < 0.05) total pixels than the OVX animals (59,096 ± 19,700 pixels vs. 138,534 ± 23,714 pixels respectively; Fig. 3).

**DISCUSSION**

One goal of the present project was to determine whether expression of the LHRH gene in the bovine brain was dependent on ovarian status of the female. We chose two groups of animals expected to have very different LHRH secretory patterns—with the OVX having a high rate of LHRH release and LUTEAL having a lower rate. Studies in sheep [21] and rats [4, 22] have demonstrated that the in vivo LHRH pulse frequency is significantly higher in gonadectomized animals than in gonad-intact animals or in those under the negative feedback effects of ovarian steroids [23, 24]. The LHRH pulse frequency is also dependent on the stage of the estrous cycle in sheep [25].

On the basis of previous experiments, we expected that the LHRH pulse generator in the OVX group would produce about 30 LH pulses per 24 h [26, 27] while the LUTEAL animals would have only 6–8 LH pulses per 24 h [28, 29]. Although it was not possible to directly measure the in vivo release of LHRH in these animals, it is likely that the 6.7-fold difference in serum LH concentrations between the two groups reflects differences in LHRH release.

As a prerequisite for this project, we had to determine whether measurement of LHRH mRNA was possible in the bovine brain, given that the bovine LHRH gene has not yet been sequenced. The deoxyoligonucleotide probe utilized for this study is 100% complementary to the human LHRH mRNA sequence and is complementary to 90% of the rat sequence. We feel confident that our probe was hybridizing to bovine LHRH mRNA because 1) sections not receiving this probe revealed no labeled neurons; 2) the use of a 32P-labeled “sense” control oligomer, identical in sequence to the LHRH mRNA sequence, yielded no hybridization signal; 3) the cells that were labeled by our probe showed an anatomical distribution similar to that of LHRH neurons identified by immunocytochemistry [6, 7]; and 4) treatment of the sections with RNAse eliminated all hybridization signal.

We found that LHRH mRNA-containing neurons were scattered throughout the diagonal band of Broca, OVLT, POA,
and the anterior hypothalamic area. Additional neurons were found in the medial septum and the nucleus of the stria terminals. The majority of the neurons were located around the OVLT and medial POA (see Fig. 1), with significantly fewer neurons observed at locations rostral and caudal to this region. Occasionally, clusters of 2–5 labeled cells were observed (Fig. 2). The anatomical distribution of our labeled neurons was not different from that described in cattle by Leshin et al. [6] or from the pattern described for the sheep [8, 9]. It was also similar to that described by Dees and McArthur [7], except that we found no labeled cells in the mediobasal hypothalamus.

Our results demonstrate that in the bovine forebrain, content of LHRH mRNA is influenced by the ovary. Heifers killed during the mid-luteal phase of the estrous cycle, that is, under the inhibitory influence of ovarian steroids (i.e., luteal progesterone), had significantly fewer detectable LHRH neurons and fewer copies of LHRH mRNA per labeled neuron. Overall, the LUTEAL phase animals had 57% fewer pixels, representing LHRH mRNA, than did the OVX females. It is likely that this measure reflects a decrease in LHRH synthetic capacity in the LUTEAL females.

Examination of cellular levels of LHRH mRNA in all evaluated labeled cells (n = 125 cells for LUTEAL, n = 189 cells for OVX) revealed that those from LUTEAL females had fewer copies of LHRH mRNA. As shown in Figure 4, cells from OVX animals had what may be a bimodal distribution of pixels/cell. In the LUTEAL females, the distribution was significantly shifted to the left of the OVX distribution, showing one mode around 2000 pixels/cell and no secondary mode.

Decreases in LHRH mRNA have also been reported in other experimental models. We have reported that blocking the estrogen-induced LH surge with an alpha-1 adrenergic antagonist (prazosin) prevented the increase in LHRH mRNA that accompanies the surge [13]. Also, prazosin decreased the LHRH mRNA by 47% in OVX rats [14]. Administration of an estrogen receptor antagonist into the POA decreased the diurnal increase in LHRH mRNA observed in estrogen-treated rats [12]. Similarly, pentobarbital administration prevented the proestrus rise in detectable LHRH cell number [10].

Ovarian steroids can either stimulate or inhibit LH release—depending on the dosage, its duration, and the time of day examined. Our results in the cow are consistent with the theory that there is a positive temporal relationship between LHRH release (as reflected by LH) and levels of LHRH mRNA in the POA. In the rat, LHRH mRNA increases in association with steroid-induced LH surges [10, 11, 13, 15], naturally occurring preovulatory LH surges [10, 30], and NMDA-induced increases in LH release [31]. Decreases in LHRH mRNA have been observed when LH concentrations are suppressed by the inhibitory effects of gonadal steroids [32, 33]. Thus, increases in LHRH gene expression may frequently be consequent to increased LHRH release.

Results presented here demonstrate that the levels of LHRH mRNA and serum LH are suppressed in the LUTEAL vs. the OVX cow. The difference in LHRH release between the two experimental groups is correlated with differences in LHRH mRNA levels. The actual cause for the decreased LHRH mRNA levels in the LUTEAL animals could be twofold: the signal that suppresses LHRH secretion might also suppress LHRH mRNA levels, or the mRNA level may be regulated by the activity of the LHRH neuron such that the decrease results from the relative quiescence of LHRH neurons during the mid-luteal phase of the cycle.

On the basis of the current results, we predict that expression of the LHRH gene would also be low during periods of chronically inhibited LHRH secretion. In domestic animals, these periods include pregnancy and lactation, seasonal anestrus, and periods of stress-induced reproductive deficits.

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