Effects of 17β-Estradiol on Distribution of Pituitary Isoforms of Luteinizing Hormone and Follicle-Stimulating Hormone during the Follicular Phase of the Bovine Estrous Cycle

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ABSTRACT

The objective of this study was to examine the influence of 17β-estradiol (E2) on distribution of LH and FSH isoforms during the follicular phase of the bovine estrous cycle prior to the preovulatory surges of LH and FSH. On Day 16 of the estrous cycle (Day 0 = estrus), intact controls (CONT; n = 4) were treated with prostaglandin F2α (PGF2α) to induce luteal regression and initiation of the follicular phase. Other cows were also treated with PGF2α and either ovariectomized (OVX; n = 5) or ovariectomized and given E2 implants (OVXE; n = 6) to mimic the pattern of increasing E2 concentrations during the follicular phase of the estrous cycle. Pituitaries were collected 40 h after treatment with PGF2α or ovariectomy (0 h). Aliquots of pituitary extracts were chromatofocused on pH 10.5–4.0 gradients. The LH resolved into thirteen isoforms (designated A-L and S, beginning with the most basic form) while FSH resolved into nine isoforms (designated I-IX, beginning with the most basic form). The percentage of LH as isoform F (elution pH = 9.32 ± 0.01) was greater (p < 0.05) in the OVX group (48.5%) than in the OVXE group (45.0%). All isoforms of the rat [4, 8, 9], sheep [5], cow [12], and human [13] LH possess significant bioactivity as measured by a rat interstitial cell bioassay. Generally in most species, the more acidic LH isoforms have lower bioactivity than either the more alkaline or acidic LH isoforms [5, 12]. Similarly, receptor binding activity of the hamster [15] and rat [16] FSH isoforms declines as the pI decreases. However, more acidic rat FSH isoforms display a longer circulatory half-life in vivo [17].

The distribution of the LH and FSH isoforms in the pituitary is altered by gonadal steroids. Distribution of the isoforms in the pituitary varies during stage of the reproductive cycle in rodents [18–20] and primates [21], and differs as a result of castration in rodents [3, 9, 14, 15], sheep [5], and cattle [12] or as a result of steroid hormone replacement in sheep [5] and cattle [12]. Interestingly, the amounts of biologically potent basic isoforms of LH increase during the preovulatory surge of gonadotropins in the rat [18] and during the midcycle surge of gonadotropins in primates [21, 22], whereas the amounts of biologically less potent, more alkaline or acidic LH isoforms remain constant throughout the entire estrous cycle [18]. These results are consistent with those of earlier studies where an increase in bioactivity of LH in circulation was observed during the preovulatory surge of LH in rats [23, 24] and during the midcycle gonadotropin surge in monkeys [25–27].

It has been reported that pattern and distribution of ovine and bovine LH [28] and rat [17], hamster [19], and human FSH [29] isoforms released from the anterior pituitary in vitro generally reflect the pituitary intracellular isoforms. According to these previous studies, changes in the endocrine milieu of animals not only alter the quantity of pituitary gonadotropins but also the quality of gonadotropins.
Thus, changes in the profile of gonadotropin isoforms could have a role in regulating reproductive function [30].

In general, the follicular phase of the bovine estrous cycle is a transition from luteal regression to the preovulatory surge of gonadotropins. The follicular phase of the estrous cycle is accompanied by increasing concentrations of 17β-estradiol (E2) after a decline in concentrations of progesterone [31,32]. During this period, mean concentrations of LH increase linearly [33,34], with an increase in frequency [34] and amplitude of LH pulses [35]. The preovulatory surge of gonadotropins occurs 50–70 h after treatment of cows with prostaglandin F2α (PGF2α) [36,37] or after the initiation of sequential E2 implantation in ovariectomized cows [32]. The distribution of intrapituitary gonadotropin isoforms has not been evaluated during the follicular phase of the bovine estrous cycle, when concentrations of E2 and LH are dramatically increased. The advantages of utilizing the cow as a model are the relatively large anterior pituitary glands, well-characterized reproductive cycles, and experimentally manipulable endocrine systems. Thus, the pattern of E2 in circulation can be experimentally manipulated by the exogenous source of E2 to mimic the endogenous pattern during the follicular phase of the bovine estrous cycle.

Therefore, we hypothesized that different patterns of gonadotropin isoforms would exist during the preovulatory surge of gonadotropins as a result of the increased concentrations of E2 that occur during the follicular phase of the estrous cycle. The change in pattern of gonadotropin isoforms may provide an appropriate stimulus for ovulation. If this is the case, the acute increase in concentrations of E2 during the follicular phase of the bovine estrous cycle or increasing exogenous E2 in the ovariectomized animal should alter the distribution of LH and FSH isoforms in the pituitary prior to the preovulatory surge of gonadotropins. This should be different from the case in the ovariectomized animal with no gonadal steroids in circulation. In addition, if E2 is the only factor regulating distribution of LH and FSH isoforms, the follicular phase intact animal and the ovariectomized animal receiving exogenous E2 should have similar patterns of isoforms.

MATERIALS AND METHODS

Experimental Protocol and Collection of Pituitaries

The experimental protocol is described in detail in a companion paper [38]. Briefly, the estrous cycles of fifteen mature beef cows (2–7 yr of age) were synchronized by two injections of PGF2α (Lutalyse® Sterile Solution; The Upjohn Co., Kalamazoo, MI) 11 days apart. On Day 16 of the following estrous cycle (Day 0 = estrus), controls (CONT; n = 4) were treated with PGF2α (Hour 0) to induce luteal regression and initiation of the follicular phase. Other cows were also treated with PGF2α and either ovariectomized (OVX; n = 5) or ovariectomized and treated with E2 implants (OVXE; n = 6) [35] at 0, 10, 15, 20, and 30 h (ovariectomy = 0 h) to mimic the pattern of increasing E2 concentrations during the follicular phase of the bovine estrous cycle. The E2 (Sigma Chemical Co., St. Louis, MO) was administered via polydimethylsiloxane intravaginal implants (3.35 mm i.d. × 4.65 mm o.d. × 13.5 cm; Dow-Corning, Midland, MI) filled with E2. Pituitaries were collected 40 h after injection of PGF2α or ovariectomy. Immediately after removal, anterior and posterior lobes of each pituitary were separated, and the anterior lobe was hemisected. Pituitary tissues were then frozen in liquid nitrogen and stored at −70°C until extracted.

Tissue Extraction

Frozen pituitary tissue was homogenized for 30 sec in 150 mM NaCl buffered with 50 mM Tris, pH 7.4, containing 0.5% (vol/vol) Triton X-100, 5 mM Na2 EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.5 mg/L leupeptin, and 200 U/ml aprotinin (1.0 ml/100 mg wet tissue weight) with a polytron homogenizer (Brinkman Instruments, Westbury, NY). Pituitary extracts were clarified by centrifugation at 100,000 × g for 1 h, aliquoted into 0.5-ml portions (equivalent to 50 mg tissue), and stored at −70°C until chromatofocusing.

Chromatofocusing

Aliquots of pituitary extract were subjected to chromatofocusing on pH 10.5–4.0 gradients. A 0.5-ml aliquot (50 mg tissue equivalent) was desalted by flow dialysis against water using 6000–8000 M cutoff membranes (Spectra/Per 1; Spectrum Medical Industries, Inc., Los Angeles, CA). Desalted extracts were supplemented to 2% (vol/vol) with Pharmalyte 8–10.5 (pH 7.0; Pharmacia/LKB Biotechnology Inc., Piscataway, NJ). Two milligrams each of cytochrome C and myoglobin were added to the sample, and the mixture was applied to a 10-ml (0.7 × 26 cm) column (Kontes, Vineland, NJ) of PBE-118 resin (Pharmacia/LKB Biotechnology Inc.) previously equilibrated in 25 mM triethylamine (pH 11.0). The pH gradient was developed (5 ml/h) with Pharmalyte 8–10.5 diluted 1:45 with distilled water and adjusted to a pH of 7.0. The elution buffer was then switched to Polybuffer 74 HCl (Pharmacia/LKB Biotechnology Inc.) diluted 1:8 with distilled water and adjusted to a pH of 4.0 with 6 N HCl. An additional seventy-five 1.5-ml fractions were collected to obtain a stable plateau near pH 7.0. The elution buffer was then switched to Polybuffer 74 HCl (Pharmacia/LKB Biotechnology Inc.) diluted 1:8 with distilled water and adjusted to a pH of 4.0 with 6 N HCl. An additional seventy-five 1.5-ml fractions were collected to reach a stable lower limiting pH of 4.0. Proteins bound to the column at this lower limiting pH were eluted with 1.0 M NaCl and collected as an additional twenty 1.5-ml fractions. The fractions were neutralized by addition of 0.15 ml of 1.1 M Tris buffer (pH = 7.0). Columns were re-equilibrated between samples with at least 50 column volumes of triethylamine. All buffers were thoroughly degassed before use and contained 1% glycerol. Recovery of immunoreactive LH and FSH from the columns averaged 89% and 85%, respectively.
RIAs

Concentrations of LH in pituitary extracts and chromatofocusing fractions were determined by RIA [39] as validated in our laboratory [40]. Intra- and interassay coefficients of variation were 3.0% and 13.2%, respectively, and assay sensitivity was 29.8 pg/ml. Concentrations of FSH in pituitary extracts and chromatofocusing fractions were determined by RIA [40, 41]. Intra- and interassay coefficients of variation were 2.4% and 12.7%, respectively, and assay sensitivity was 74.1 pg/ml.

Statistical Analysis

The effects of treatment on pituitary hormone characteristics were analyzed by one-way analysis of variance [42] according to the General Linear Models procedure of SAS [43]. Differences in treatment means were detected by Duncan's New Multiple Range test [42]. Percentage values were subjected to arc sine transformations (arc sine of the square root of the proportion corrected to an angle between 0 and 90°) before analysis. A probability of less than 0.05 was considered statistically significant.

RESULTS

Concentrations of LH, FSH, and E2 in Circulation

Concentrations of these hormones are described in detail in the companion paper [38]. Briefly, concentrations of E2 in the OVX cows remained low after ovariectomy through 40 h, at the end of which pituitaries were collected. Cows from the CONT and OVXE groups had increasing concentrations of E2 in circulation during the 40-h period, and the pattern of increasing E2 did not differ between these groups. Therefore, the experimental protocol for administration of E2 implants (OVXE) used in the present study effectively mimicked the pattern of increasing concentrations of E2 in intact animals (CONT) during the follicular phase of the bovine estrus cycle [38].

Mean concentrations of LH were greater (p < 0.05) in cows from the OVXE group during 40 h of blood collections (see companion protocol for blood collections) after injection of PGF2α, ovariectomy, and/or initiation of E2 implants compared to LH concentrations in the CONT group. Mean concentrations of LH were similar (p > 0.10) among cows from the OVX and CONT groups during 40 h of blood collections. Mean concentrations of FSH were greater (p < 0.01) in cows from OVX and OVXE groups during 40 h of blood collections after initiation of the treatment compared to those from the CONT group, where mean concentrations of FSH remained similar (p > 0.10) in cows from the OVX and OVXE groups. Mean pituitary weights and pituitary contents of LH and FSH were similar (p > 0.10) among all groups [38].

LH Isoforms

The LH in each pituitary extract resolved into thirteen isoforms when chromatofocused over a pH 10.5-4.0 gra-
The isofoms of LH were coded with letters A-L and S, beginning with the most basic form. Twelve isoforms eluted in the separating pH range of the column, and the thirteenth isoform was bound to the column at the lower limiting pH. The predominant LH isoforms that eluted in the basic pH range were F and G, and those forms accounted for at least 60% of the immunoreactive LH in the pituitary.

Differences existed in distribution of LH isoforms in pituitaries among cows from the OVX and OVXE groups. The percentage of LH as isoform F (elution pH = 9.32 ± 0.01) was greater (p < 0.05) in the OVX group (48.5%) than in the OVXE group (45.0%). LH isoforms I (elution pH = 6.98 ± 0.01) and J (elution pH = 6.48 ± 0.01) were more abundant (p < 0.05) in cows from the OVXE (2.3 and 5.8%, respectively) than from the OVX group (1.4 and 3.7%, respectively). Distribution of LH isoforms in cows from the CONT group did not differ (p > 0.10) from either the OVX or OVXE groups. The quantity of each LH isoform (data not shown) and the pituitary content of FSH [38] were not different (p > 0.10) among treatments.

**DISCUSSION**

The distribution of gonadotropin isoforms is thought to reflect the endocrine status of the animal. The experimental protocol used in the present study provided a means for examining the effects of E2 on distribution of gonadotropin isoforms during the follicular phase of the bovine estrous cycle. Cows from the OVX group were used as a negative control (no gonadal steroids), while cows in the OVXE group were administered E2 implants in a manner that mimicked the pattern of increasing concentrations of E2 during the follicular phase of the bovine estrous cycle. Comparable concentrations of E2 were observed in the CONT and OVXE groups. Furthermore, none of the animals initiated the preovulatory (CONT) or preovulatory-like (OVXE) LH surge before collection of pituitaries in the present study [38].

Gonadotropin isoforms were analyzed by chromatofocusing on pH 10.5–4.0 gradients. These pH gradients were extended below 7.0 to 4.0, which allowed for characterization of both LH and FSH isoforms with a single chromatographic separation for each pituitary extract [12]. The LH in extracts of bovine pituitaries resolved into thirteen isoforms when chromatofocused on pH 10.5–4.0 gradients [12]. The distribution pattern of LH isoforms observed in the present study corresponded closely to previous results obtained for sheep [5] and cattle [12, 28, 44] in the basic portion of the gradient (pH 10.5–7.0), and also corresponded to results obtained for sheep [45] and cattle [12] in the acidic portion of the gradient (pH 7.0–4.0). The predominant LH isoforms that eluted in the basic pH range were F and G, and those forms accounted for at least 60% of the immunoreactive LH in the pituitary. The present results were thus

### Table 1. Distribution of bovine LH isoforms in anterior pituitary tissue.

<table>
<thead>
<tr>
<th>Isohormone</th>
<th>(Elution pH)</th>
<th>CONT</th>
<th>OVXE</th>
<th>OVX</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(11.15 ± 0.03)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>B</td>
<td>(10.09 ± 0.01)</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>C</td>
<td>(9.68 ± 0.01)</td>
<td>0.5</td>
<td>0.7</td>
<td>0.9</td>
<td>0.16</td>
</tr>
<tr>
<td>D</td>
<td>(9.54 ± 0.01)</td>
<td>2.9</td>
<td>2.6</td>
<td>3.3</td>
<td>0.40</td>
</tr>
<tr>
<td>E</td>
<td>(9.44 ± 0.01)</td>
<td>11.5</td>
<td>11.8</td>
<td>11.6</td>
<td>0.71</td>
</tr>
<tr>
<td>F</td>
<td>(9.32 ± 0.01)</td>
<td>46.8</td>
<td>45.0</td>
<td>48.5</td>
<td>0.85</td>
</tr>
<tr>
<td>G</td>
<td>(8.78 ± 0.03)</td>
<td>23.1</td>
<td>22.4</td>
<td>23.2</td>
<td>0.94</td>
</tr>
<tr>
<td>H</td>
<td>(7.51 ± 0.02)</td>
<td>2.6</td>
<td>2.4</td>
<td>1.8</td>
<td>0.36</td>
</tr>
<tr>
<td>I</td>
<td>(6.98 ± 0.01)</td>
<td>1.8</td>
<td>2.3</td>
<td>1.4</td>
<td>0.21</td>
</tr>
<tr>
<td>J</td>
<td>(6.48 ± 0.01)</td>
<td>5.4</td>
<td>5.8</td>
<td>3.7</td>
<td>0.58</td>
</tr>
<tr>
<td>K</td>
<td>(5.48 ± 0.02)</td>
<td>3.7</td>
<td>4.9</td>
<td>4.0</td>
<td>0.65</td>
</tr>
<tr>
<td>L</td>
<td>(4.27 ± 0.02)</td>
<td>0.9</td>
<td>1.2</td>
<td>0.8</td>
<td>0.14</td>
</tr>
<tr>
<td>S</td>
<td>(&lt; 4.0)</td>
<td>0.5</td>
<td>0.7</td>
<td>0.5</td>
<td>0.07</td>
</tr>
</tbody>
</table>

1 Mean percentages for each isoform.
2 CONT: control cows were treated with PGF2α (n = 4); OVXE: ovariectomized and treated with E2 (n = 6); OVX: ovariectomized (n = 5); pituitaries collected 40 h after treatment with PGF2α, ovariectomy or initiation of E2 treatment (0 h).

Means identified by different superscript letters within rows differ (p < 0.05).
ESTRADIOL AND DISTRIBUTION OF BOVINE GONADOTROPIN ISOFORMS

similar to those observed in the previous study using cattle [12].

The FSH in pituitary extracts resolved into nine isoforms when chromatofocused on pH 10.5–4.0 gradients. The distribution pattern of FSH isoforms observed in the present study corresponded closely to previous results obtained for cattle [12]. Seven isoforms eluted in the separating pH range of the column, and two were bound to the column at the lower limiting pH. We previously reported FSH isoform VIII in bovine pituitary extracts, which was not tightly bound to the column and eluted immediately after 1 M NaCl was applied to the column [12]; however, isoform VIII constituted less than 1% of the immunoreactive FSH in all pituitary extracts in the present study. This probably was the result of the extended plateau at the lower limiting pH of 4.0 in the present study. The most acidic isoform, X, was tightly bound to the column and eluted as the 1 M NaCl front reached the bottom of the column; this isoform accounted for at least 23% of the immunoreactive FSH in the pituitary. Isoform IX may contain multiple components that could be resolved by an elution pH of less than 4.0 or by alternate procedures.

Analysis of isoforms of ovine FSH by chromatofocusing on pH 7.0–4.0 gradients also yields nine isoforms, in which the most basic ovine FSH isoform elutes as a flow-through peak indicating an elution pH of greater than 7.0 [46]. Similar to our previous study [12], in the present study the most basic isoform of bovine FSH, I, was observed as a distinct peak in cows of the OVX and OVXE groups. Interestingly, this peak was not observed in the intact (CONT) cows. However, neither the percentage nor quantity of FSH isoform I differed among treatments.

We hypothesized that a different composition of gonadotropin isoforms would exist in the pituitary during the period prior to the preovulatory surge of gonadotropins, which would provide an appropriate stimulus for ovulation in the bovine. The distributions of LH isoforms in the pituitaries of all groups were similar in that none of the thirteen observed isoforms constituted biologically meaningful changes in the distribution of immunoreactive LH. However, differences between cows from the OVX and OVXE groups in isoforms F, I, and J were observed. A greater percentage of LH was present as isoform F in the OVX than in the OVXE group (48.5% and 45.0%, respectively), whereas the less prevalent isoforms I and J were more abundant in cows from the OVXE than the OVX group (1.4 and 2.3%; 3.7 and 5.8%, respectively). The distribution of all LH isoforms in cows from the CONT group was not different from that in either the OVX or OVXE groups. Nonetheless, differences in the distribution of LH isoforms in the pituitary among treatments were relatively small.

It has been reported that castration significantly increased the percentage of basic isoforms in rats [47] and sheep [5, 48], and castration or ovariectomy followed by treatment with E₂ resulted in a significantly greater percentage of acidic isoforms of LH in sheep [5] and cattle [12]. The experimental protocol used in the present study allowed for manipulation of the pattern of E₂ in circulation of animals in a short-term period analogous to the hormonal milieu observed during the follicular phase of the

FIG. 2. Representative chromatofocusing elution profile of immunoreactive FSH in the pituitary. A 0.5-ml aliquot of pituitary extract (50-mg tissue equivalents) was chromatofocused on a pH 10.5–4.0 gradient. Each peak was coded with Roman numerals beginning with the most basic form (I through VIII). Proteins bound to column at lower limiting pH were eluted with 1.0 M NaCl and designated peaks VIII and IX. Pituitaries were collected 40 h after treatment with PGF₉₀, ovariectomy, or initiation of E₂ treatment (0 h).
estrous cycle in the cow. This experimental protocol resulted in slight shifts in the distribution of LH isoforms. However, the differences observed in the present study were not of the same magnitude as reported previously in rats [47], sheep [5, 48] and cattle [12]. It is likely that long-term gonadectomy has a more pronounced effect on distribution of LH isoforms. The increase in the percentage of either basic or acidic isoforms observed in previous studies [5,12,47,48] might have developed over a longer period of time, whereas short-term changes in the hormonal milieu would be required to change the pattern of LH isoforms during the estrous cycle. Therefore, the experimental protocols that include long-term gonadectomy and/or steroid hormone replacement, which resulted in significant changes in the pattern of LH isoforms, are not of the same magnitude as reported previously in rats [18] and during the midcycle surge in primates [21, 22], while biologically less potent, more alkaline or acidic rat LH isoforms remain constant throughout the entire estrous cycle [18]. These results may indicate that there are species differences in gonadotropin heterogeneity. Therefore, changes in LH and FSH heterogeneity do not appear to be a significant mechanism in regulating reproductive function in sheep and cattle.

In summary, removal of the ovary (OVX) slightly increased the percentage of the basic LH isoform F over that in the OVXE group, whereas removal of the ovary and administration of E2 in a pattern that mimicked the increase in concentrations of E2 during the follicular phase of the bovine estrous cycle (OVXE) resulted in a slight increase in the percentage of the acidic LH isoforms (1 and J) over the percentage in the OVX group. However, distribution of LH isoforms in intact cows (CONT) during the follicular phase of the estrous cycle did not differ from that of either the OVX or OVXE groups. There was no influence of ovarioectomy or treatment with E2 on distribution of FSH isoforms in the pituitary. Therefore, we reject our hypothesis because a different distribution pattern of gonadotropin isoforms does not exist in the pituitary during the follicular phase of the bovine estrous cycle just prior to the preovulatory surge of gonadotropins.

**ACKNOWLEDGMENTS**

We thank Ken Pearson, Georgette Caddy, and Deb Clopton for assistance with laboratory analysis, Karl Moline, Jeff Bergman, and Bob Broweleit for management support.

### TABLE 2. Distribution of bovine FSH isoforms in anterior pituitary tissue.1

<table>
<thead>
<tr>
<th>Isohormone (Elution pH)</th>
<th>CONT</th>
<th>OVXE</th>
<th>OVX</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (9.07 ± 0.003)</td>
<td>9.5</td>
<td>8.6</td>
<td>8.8</td>
<td>0.89</td>
</tr>
<tr>
<td>II (6.46 ± 0.009)</td>
<td>5.5</td>
<td>5.9</td>
<td>3.8</td>
<td>0.93</td>
</tr>
<tr>
<td>III (5.30 ± 0.023)</td>
<td>10.2</td>
<td>8.0</td>
<td>8.6</td>
<td>0.85</td>
</tr>
<tr>
<td>IV-VI (4.66 ± 0.017)</td>
<td>29.6</td>
<td>29.9</td>
<td>33.4</td>
<td>1.34</td>
</tr>
<tr>
<td>VII (4.06 ± 0.018)</td>
<td>21.3</td>
<td>22.9</td>
<td>21.9</td>
<td>1.09</td>
</tr>
<tr>
<td>VIII (4.59 ± 0.026)</td>
<td>0.6</td>
<td>0.9</td>
<td>0.6</td>
<td>0.17</td>
</tr>
<tr>
<td>IX (&lt; 4.0)</td>
<td>23.3</td>
<td>23.8</td>
<td>23.0</td>
<td>1.59</td>
</tr>
</tbody>
</table>

1 Mean percentages for each isoform.
2 CONT: control cows were treated with PGF2α (n = 4); OVXE: ovarioctomized and treated with E2 (n = 6); OVX: ovarioctomized (n = 5); pituitaries collected 40 h after treatment with PGF2α, ovarioectomy or initiation of E2 treatment (0 h).
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