Effect of Long-Term Infusion with Recombinant Growth Hormone-Releasing Factor and Recombinant Bovine Somatotropin on Development and Function of Dominant Follicles and Corpora Lutea in Holstein Cows

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ABSTRACT

The objective of this study was to evaluate the effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bovine somatotropin (rbST) on growth and function of the first-wave dominant follicle and corpus luteum. Primiparous Holstein cows (117 d postpartum) were infused with 12 mg/d of rGRF (n = 10) or 29 mg/d of rbST (n = 10) for 63 d, and non-infused cows (n = 10) were controls. At slaughter on d 5 of an estrous cycle, blood and ovaries were collected and data from cows with a corpus luteum were analyzed (control, n = 8; rGRF, n = 5; rbST, n = 6). Treatment with rGRF or rbST increased somatotropin (ST) and IGF-I in serum similarly compared with controls. In contrast, rbST-treated cows had higher concentrations of ST in follicular fluid (FF) compared with rGRF-treated and control cows. In addition, rbST, but not rGRF, increased the number and decreased the size of estrogen-active follicles (EA; estradiol > progesterone concentrations in FF), increased the abundance of IGF binding proteins-2, -3, and -4 in FF from EA follicles, and increased the number but decreased the size of corpora lutea and decreased concentration of progesterone in serum compared with controls. Based on these results, we concluded that long-term infusion of rbST alters growth and function of the first-wave dominant follicle and the corpus luteum in cattle.

(Key words: bovine somatotropin, dominant follicle, corpus luteum, cow)

Abbreviation key: CL = corpus lutea, EA = estrogen-active, EI = estrogen-inactive, FF = follicular fluid, IGFBP = IGF-binding protein, rGRF = recombinant growth hormone-releasing factor, rbST = recombinant bST, ST = somatotropin.

INTRODUCTION

Growth hormone-releasing factor (GRF) and bovine somatotropin, which increase milk production in dairy cows (2, 3), also alter ovarian function. For example, GRF analogs increase follicle size and intrafollicular concentrations of progesterone in dairy cows (61) and stimulate the proliferation of bovine granulosa cells (60). Administration of recombinant bST (rbST) increases the number (22) and size of follicles (37, 41), growth of (39) and progesterone production by the corpus luteum [CL, (16, 39, 56)], incidence of twinning (8, 15), the interval from calving to conception and calving intervals (43), and reduces expression of estrus (15, 36). Because somatotropin (ST) receptors are in the CL (38) and in granulosa cells from different size follicles (34), the effects of rbST on follicular growth and function may be direct. Moreover, abnormal ST receptor function in miniature Brahman cattle results in small body size, fewer ovarian follicles, and smaller size of dominant follicles and CL (6). Together, these data suggest that GRF or rbST can modify follicular growth and function and subsequent growth and function of the corpus luteum.

During the bovine estrous cycle, two or three waves of ovarian follicles develop. During each wave, a single dominant follicle develops and either ovulates or undergoes atresia (19, 28, 52). Long-term infusion effects of recombinant bovine GRF (rGRF) or rbST on development and function of a dominant follicle and CL in dairy cows have not been examined. Therefore, this study tested the hypothesis that rGRF or rbST treatment alters growth and function of the dominant follicle and corpus luteum in dairy cows. Whether follicular dominance is altered was monitored by examining the effects of rGRF or rbST on a) the occurrence of ovulation after PGF2α treatment, b) ovulation rate, c) luteal function,
and d) follicular hierarchy and function during development of the first-wave dominant follicle.

MATERIALS AND METHODS

Cows and Blood and Tissue Collection

Thirty primiparous Holstein cows weighing 523 ± 7 kg were housed in tie stalls and fed a total mixed ration (3, 47). Eight weeks before treatments began, an infusion catheter (VETport; Thermedics, Woburn, MA) was implanted surgically into the left jugular vein of cows assigned to rGRF and rbST groups, as described by Binelli et al. (3). Cows were infused beginning 117 d postpartum (d 1 of treatment) with rGRF (12 mg/d; Leu27, Homoserine45-bGRF1-45 lactone; Pharmacia and Upjohn Inc., Kalamazoo, MI; n = 10 cows) or rbST (29 mg/d; Somavubove; Pharmacia and Upjohn Inc., Kalamazoo, MI; n = 10 cows) for 63 d, and 10 cows were untreated controls [See Binelli et al. (3) for further details]. Prostaglandin F2α (Lutalyse; Pharmacia and Upjohn Inc., Kalamazoo, MI; n = 10 cows) for 63 d, and 10 cows were untreated controls [See Binelli et al. (3) for further details]. Prostaglandin F2α (Lutalyse; Pharmacia and Upjohn Inc., Kalamazoo, MI) was injected into all cows on d 43 and 54 of treatment to synchronize estrus. Cows were not observed for estrus because infusion pumps were attached to each cow’s back, and cows were tied in stalls except during milking. On d 57 of treatment, blood was sampled at 20-min intervals for 6 h, and LH and FSH were measured. Cows were not fasted before slaughter on d 63 of treatment (180 d postpartum). At slaughter, one 50-ml blood sample was collected from trunk blood, and the ovaries were collected and weighed. Because blood samples were not taken before PGF2α injection and during luteal regression, the stage of the estrous cycle at slaughter was estimated based on the appearance of the corpus luteum (26) to verify that at the time of ovary collection cows were between d 5 to 7 of the estrous cycle (9 d after the last PGF2α injection). Number and weight of corpora lutea, number and size of follicles ≥5 mm in diameter, and size of five follicles ≤5 mm in diameter were individually recorded. Follicular fluid (FF) was collected from all follicles measured. Serum and FF were stored at −20°C until assayed for concentration of ST, IGF-I, IGF-binding protein (IGFBP), estradiol, progesterone, and androstenedione.

Ratios of concentration of estradiol to progesterone in follicular fluid were used to classify follicles into two different stages of differentiation: estrogen-active (EA; estradiol > progesterone) and estrogen-inactive (EI; progesterone > estradiol, (27)).

Radioimmunoassays

Progesterone in unextracted serum and FF, and estradiol in unextracted FF were determined with commercially available radioimmunoassay (RIA) kits (Diagnostic Products Corporation, Los Angeles, CA) as validated by Ireland et al. (29). Sensitivity of the progesterone assay was 0.1 ng/ml, and intra- and inter-assay coefficients of variation (CV) were 5 and 9%, respectively. The sensitivity of the estradiol assay was 0.5 pg/ml, and intra- and inter-assay CV were 6 and 7%, respectively. Estradiol in ether-extracted serum was quantified in a single assay with a different RIA kit (Serono Diagnostics, Allentown, PA) with modifications as validated by Prendiville et al. (51). The sensitivity of the estradiol assay was 0.19 pg/ml, and intra-assay CV was 4%. Androstenedione in FF was quantified by a solid-phase RIA kit (Diagnostic Products Corporation, Los Angeles, CA) per manufacturer’s instructions with the following modifications. Five microliters of FF was extracted (86% extraction efficiency) once with 1 ml of diethyl ether. After evaporation of solvent for 60 min at 37°C, residues were dissolved in 500 μl of buffer supplied with the kit, incubated at 37°C for 30 min, and then duplicate 200-μl aliquots were assayed. Increasing volumes (0.4 to 40 μl) of ether-extracted FF decreased binding parallel to the standard curve (data not shown). Sensitivity of the androstenedione assay was 20 pg/ml and intra-assay CV was 6%.

Insulin-like growth factor-I was quantified by RIA (57) in formic acid-ethanol extracted serum and FF (86% extraction efficiency). Recombinant human IGF-I (DGR012; Bachem, Inc. Torrance, CA) was radioiodinated with chloramine T, and the international reference for human IGF-I was used as standard. Antiserum against human IGF-I (NIH-AB UBK487, kindly supplied by L. Underwood, University of North Carolina, Chapel Hill, NC) was diluted 1:10,000 in assay buffer (0.3 M phosphate buffer, 0.01 M EDTA, pH 7.5). Sensitivity of the assay was 12.5 pg/tube, and intra-assay CV for serum and FF were 6 and 5%, respectively. Cross-reactivity with IGF-II was <0.5%.

Somatotropin in serum and FF were quantified by a heterologous RIA (17) with the following modifications. Recombinant bovine ST (U-72104; Pharmac and Upjohn Inc., Kalamazoo, MI) was used as standard or radioiodinated with chloramine T (17). Antiserum to ovine ST (NIADDK-anti-oGH-2) was diluted 1:80,000 in 1:400 normal rabbit serum (Gibco Laboratories, Madison, WI) in 0.01 M PBS (with 0.05 M EDTA, pH 7.0). Assay buffer was 1% BSA in 0.01 M PBS, pH 7.0. Duplicate 100 μl of rbST standards (0.001 to 1.0 ng), serum or follicular fluid (diluted 1:20 in assay buffer) were added to 12 × 75 mm glass tubes followed by 50 μl of antiserum. Each tube was vortexed, incubated for 24 h at 4°C, and then 125I-rbST (50 μl, 0.011 μCi) was added. Tubes were incubated for an additional 48 h at 4°C. One hundred microliters of S. aureus protein A
Concentration of FSH in serum was determined by a validated heterologous RIA (20); 125I-ovine FSH (USDA-oFSH-19-SIAFP-I-2), bovine FSH (USDA-bFSH-I-2) as standard, and rabbit anti-ovine FSH-1 (AFP-C5288113) were used for antiserum. Sensitivity of the assay was 30 pg/ml and intra-assay CV was 9%. Cross-reactivity with EHC-bLH-1 was <1% (20). Concentration of LH was determined by RIA (42) with bovine LH (USDA-bLH-I-2, AFP-5500) for radioiodination and as standard, and a monoclonal anti-bovine LH antibody (B-518B7, kindly donated by Dr. J. F. Roser, University of California, Davis, CA). Sensitivity of the assay was 95 pg/ml and intra-assay CV was 11%. Cross-reactivity with bovine FSH was <0.04% (42).

**Ligand Blot Analysis**

Recombinant human IGF-I (5 μg; H-5555; Bachem, Torrance, CA) was iodinated with 1 mCi Na125I (NEZ-033H; New England Nuclear, Boston, MA) and 50 μg of Iodogen (Pierce, Rockford, IL) for 10 min (11). Iodinated IGF-I was purified on a Sephadex G-25 column (PD-10, 5 × 1.6 cm prepacked column; Pharmacia, Piscataway, NJ) previously equilibrated with 25 ml of column buffer (0.01 M Na2HPO4, pH 7.2, 3% BSA), and aliquots were stored at 4°C until used. Specific activity was 97.7 μCi/μg protein. Radiolabeled IGF-I was diluted to 200,000 cpm/ml (0.11 μCi/ml) before ligand blot analysis.

Follicular fluid samples (25 μg of protein/lane; n = 25 total follicles) from EA follicles were subjected to 15% SDS-PAGE (n = 5 gels, (35)) with a mini-gel apparatus (Mini-Protein II Dual Slab Cell; Bio-Rad, Richmond, CA), according to manufacturer’s instructions. Proteins were transferred to an Immobilon P membrane with a Bio-Rad Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA) according to manufacturer’s instructions and processed for ligand blot analysis by procedures described by Good et al. (24). Molecular weight estimates were based on protein standards (Bio-Rad, Richmond, CA) after silver staining, and intensities of bands after ligand blotting were determined by the Molecular Analyst Software for Bio-Rad GS 250 Molecular Imager after exposure of blots for 82 h to a Bio-Rad GS 250 Imaging Screen-BI (Bio-Rad, Richmond, CA), as reported by Good et al. (24).

**Statistical Analysis**

Two cows from each treatment group were removed from analysis because PGF2α was injected only once, so ovarian status at slaughter was not synchronized with other cows. In addition, two cows infused with rbST, and three cows infused with rGRF did not have corpora lutea at slaughter; thus these data were not analyzed. Data from the cows with corpora lutea (control, n = 8; rbST, n = 6; rGRF, n = 5) were analyzed by the general linear model procedure of SAS (53). The concentration of serum hormones, number and weight of corpora lutea, number of follicles, and number of estrogen-active follicles were analyzed as a randomized block design (18). The model included the effects of treatment, block and residual. Concentration of hormones in follicular fluid, diameter of follicles >5 mm, and diameter of estrogen-active follicles were analyzed as a split block design with repeated measurements in space (18). The model included the effects of treatment, block, treatment by block, follicular class (ratio of estradiol to progesterone), treatment by follicular class, block by follicular class and residual. An outlier test (18) was used to test values disparate from the mean of a given variable. The significance of treatment and block effects was tested with treatment by block as the error term. The amount of IGFBP in follicular fluid from estrogen-active follicles was analyzed by ANOVA (18). Means were compared by the Bonferroni-t test (53). Differences in the proportion of cows that responded to PGF2α, treatment, cows with more than five follicles, and cows with estrogen-active follicles within each follicle class were analyzed by χ2. Unless stated otherwise, effects with P < 0.05 were considered significant. Values of estradiol, progesterone, androstenedione, IGF-I and ST in follicular fluid were log transformed to satisfy assumptions of normally distributed errors before statistical analysis, but actual values are reported.

**RESULTS**

**Occurrence of Ovulation after Prostaglandin F2α Treatment**

The number of cows with a CL after two PGF2α injections tended (P < 0.10) to be lower in the rGRF- and rbST-treated groups compared with controls (69 vs. 100%). Cows that ovulated in response to synchronization had a new CL, whereas those that did not respond did not have a CL. Each cow without a CL had at least one corpus albicans and several follicles indicating that they had undergone estrous cycles. For cows with a CL, the average (±SEM) estimated day of the estrous cycle at time of slaughter for controls, rGRF- and rbST-treated groups was 5.4 ± 0.4, 4.3 ± 0.5 and 5.2 ± 0.4 d, respectively.
TABLE 1. Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bST (rbST) on serum hormone concentrations in Holstein cows.1

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control (n = 8)</th>
<th>rGRF (n = 5)</th>
<th>rbST (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>SEM</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>Somatotropin, ng/ml</td>
<td>12.8</td>
<td>2.3</td>
<td>18.7a</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>238b</td>
<td>8</td>
<td>520*</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>8.0</td>
<td>0.7</td>
<td>12.1</td>
</tr>
<tr>
<td>Progesterone, ng/ml</td>
<td>2.2a</td>
<td>0.2</td>
<td>1.4ab</td>
</tr>
<tr>
<td>LH, ng/ml</td>
<td>0.4</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>FSH, ng/ml</td>
<td>1.1</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a,bMeans in a row with unlike superscripts differ (P < 0.05).
1Cows were infused with rGRF or rbST from 117 to 180 d postpartum. The concentration of hormones was determined for serum collected on d 5 of a estrous cycle at slaughter.

respectively. Treatments did not affect estimated day of the estrous cycle, thus day of the estrous cycle will hereafter be referred to as d 5 postestrus. Note, the estimated day of the estrous cycle in this study was similar to expected day of the cycle based on previous studies that evaluated effects of two injections of PGF$_2$α spaced 11 d apart on synchronization of estrus in postpartum dairy cows (48).

Concentration of Hormones in Serum

The concentration of LH or FSH measured at 20-min intervals for 6 h on d 57 of treatment (overall means = LH: 0.52 ± 0.20, 0.78 ± 0.25, and 0.65 ± 0.14 ng/ml, and FSH: 1.05 ± 0.07, 0.94 ± 0.06, and 1.29 ± 0.16 ng/ml for control, rGRF-, and rbST-treated cows, respectively) or at slaughter after d 63 of treatment (Table 1) did not differ among treatment groups.

After 63 d of treatment, concentrations of ST and IGF-I in serum were greater (P < 0.05) in rGRF- and rbST-treated cows compared with controls (Table 1). In addition, ST concentrations tended (P < 0.10) to be greater in rbST-treated cows compared with rGRF-treated cows. Even though all cows were on d 5 of the estrous cycle, concentration of progesterone in rbST-treated cows was lower (P < 0.05) than in controls. Concentrations of estradiol did not differ among treatment groups after 63 d of treatment.

Corpora Lutea

The number of CL was greater (P < 0.05), but weight per CL was lower in rbST-treated cows compared with controls (P < 0.05) or rGRF-treated (P < 0.10) cows (Table 2). However, total amount of luteal tissue per cow did not differ among treatments (6.2 ± 0.5, 4.8 ± 1.1, and 4.9 ± 0.9 g for controls, rGRF-, and rbST-treated cows, respectively).

Follicles

Number of follicles per cow and diameter of follicles >5 mm did not differ (P > 0.10) among treatments (Table 2). However, a greater proportion of cows in the rbST group (87%) tended (P < 0.10) to have five or more follicles >5 mm in diameter compared with controls (37%) or rGRF-treated (25%) cows. The size of follicles and volume of follicular fluid from follicles ≤5 mm in diameter did not differ among treatments (data not shown).

The concentration of ST in follicular fluid in rGRF- and rbST-treated cows was greater (P < 0.05) compared with controls (Table 3). In addition, ST concentration was greater (P < 0.05) in rbST- compared with rGRF-treated cows. Androstenedione in FF of control cows was higher (P < 0.05) compared with rGRF-treated cows. Concentrations of IGF-I, estradiol, and progesterone did not differ among treatments.

Estrogen-Active and Estrogen-Inactive Follicles

The number of EA follicles per cow was greater (P < 0.05) in rGRF- and rbST-treated cows compared with controls (Table 2). In addition, ST concentrations tended (P < 0.10) to be greater in rbST-treated cows compared with rGRF-treated cows. Even though all cows were on d 5 of the estrous cycle, concentration of progesterone in rbST-treated cows was lower (P < 0.05) than in controls. Concentrations of estradiol and progesterone did not differ among treatment groups after 63 d of treatment.

Ligand blot analysis was used to evaluate whether rGRF or rbST altered the amount of IGFBP in follicular
BOVINE SOMATOTROPIN AND FOLLICULAR DOMINANCE

TABLE 2. Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bST (rbST) on number and weight of corpora lutea (CL) and number and diameter of follicles >5 mm in Holstein cows.1

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>rGRF (n = 5)</th>
<th>rbST (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SEM</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>CL per cow, no.</td>
<td>1.0b</td>
<td>0</td>
<td>1.0b</td>
</tr>
<tr>
<td>Weight of CL, g</td>
<td>6.2a</td>
<td>0.5</td>
<td>4.8a</td>
</tr>
<tr>
<td>Follicles per cow, no.</td>
<td>4.0</td>
<td>0.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Follicular diameter, mm</td>
<td>10.7</td>
<td>0.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Estrogen-active2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicles per cow, no.</td>
<td>0.9b</td>
<td>0.2</td>
<td>1.0ab</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>16.1a</td>
<td>1.2</td>
<td>15.2ab</td>
</tr>
</tbody>
</table>

\( a,b \)Means in a row with unlike superscripts differ (\( P < 0.05 \)).

1Cows were infused with rGRF or rbST from 117 to 180 d postpartum. Measurements were made on d 5 of an estrous cycle at slaughter.

2Estrogen-active follicles have more estradiol than progesterone in follicular fluid.

fluid from estrogen-active follicles. Amounts of IGFBP in estrogen-inactive follicles were not evaluated because there were no effects of treatments on these follicles. The administration of rbST increased (\( P < 0.05 \)) the amount of IGFBP-2, -3, and -4 in follicular fluid from EA follicles compared with controls and rGRF-treated cows (Figure 1). A representative ligand blot of IGFBP detected in follicular fluid from estrogen-active follicles of rGRF-, rbST-treated and control cows is shown in Figure 2.

**DISCUSSION**

The most significant findings in the present study are that: a) long-term infusion (63 d) of rbST modifies follicular and corpus luteum growth and function, and b) rGRF treatment did not alter growth and function of follicles and corpus luteum, despite an increase in serum and intrafollicular ST concentrations, and a decrease in androstenedione levels in follicular fluid. In support of the alternative effects of rbST on the dominant follicle, others report that rbST: a) increases the number of small [2 to 5 mm, (22)], medium [6 to 9 mm, (10, 31)], and large [10 to 15 mm, (10)] follicles, b) decreases the size of the largest follicles (10, 37), c) increases the size of second largest follicles (10, 37, 40), d) increases (10) or decreases (37) estradiol concentration in serum during growth of preovulatory follicles, e) hastens the emergence of the second-wave dominant follicle growth (31, 39), and f) increases twinning (8, 15). Moreover, rbST increases (39) or decreases (31) CL size, and increases (16, 39, 56) or decreases (31, 69) progesterone concentration in serum. Taken together, administration of rbST has effects on ovarian events. Of practical significance, alterations in growth and function of dominant follicles and the corpus luteum may explain the decrease in reproductive efficiency after rbST treatment reported in several experiments (5, 8, 14, 15, 33, 36, 43, 45, 69).

In stark contrast to previous studies and results of the present experiment, there are reports that show that rbST treatment does not alter follicular or luteal development and function in dairy cows (10, 22, 37, 40, 56, 64, 70). Although the reason for conflicting results among studies is unknown, confounding factors such as parity, stage of lactation, energy balance, concentration of ST achieved during treatments, stage of the estrous cycle, length of treatments or type of injections

TABLE 3. Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bST (rbST) on hormone concentrations in follicular fluid of Holstein cows.1

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control (n = 31)</th>
<th>rGRF (n = 21)</th>
<th>rbST (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SEM</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>Somatotropin</td>
<td>5.9a</td>
<td>0.9</td>
<td>14.9b</td>
</tr>
<tr>
<td>IGF-I</td>
<td>137</td>
<td>13</td>
<td>125</td>
</tr>
<tr>
<td>Estradiol</td>
<td>48</td>
<td>12</td>
<td>35</td>
</tr>
<tr>
<td>Progesterone</td>
<td>53</td>
<td>12</td>
<td>57</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>64a</td>
<td>19</td>
<td>19b</td>
</tr>
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\( a,b \)Means in a row with unlike superscripts differ (\( P < 0.05 \)).

1Cows were infused with rGRF or rbST from 117 to 180 d postpartum. Concentration of hormones was determined in follicular fluid from follicles collected on d 5 of an estrous cycle at slaughter.
TABLE 4. Effects of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bST (rbST) on hormone concentrations in follicular fluid from follicles classified as estrogen active (EA) or estrogen inactive (EI) in Holstein cows.1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>rGRF</th>
<th>rbST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x SEM</td>
<td>x SEM</td>
<td>x SEM</td>
</tr>
<tr>
<td>EA follicles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somatotropin, ng/ml</td>
<td>5.2b 1.2</td>
<td>14.5ab 3.3</td>
<td>24.4a 1.5</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>127 23</td>
<td>142 34</td>
<td>170 24</td>
</tr>
<tr>
<td>Estradiol, ng/ml</td>
<td>209 55</td>
<td>146 56</td>
<td>177 41</td>
</tr>
<tr>
<td>Progesterone, ng/ml</td>
<td>24 6</td>
<td>20 4</td>
<td>25 4</td>
</tr>
<tr>
<td>Androstenedione, ng/ml</td>
<td>122a 68</td>
<td>21b 13</td>
<td>43ab 11</td>
</tr>
<tr>
<td>EI follicles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somatotropin, ng/ml</td>
<td>5.2c 1.2</td>
<td>15 b 1.7</td>
<td>23.6a 1.4</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>140 16</td>
<td>121 7</td>
<td>176 22</td>
</tr>
<tr>
<td>Estradiol, ng/ml</td>
<td>1.8 0.9</td>
<td>3.9 1.3</td>
<td>1.4 0.5</td>
</tr>
<tr>
<td>Progesterone, ng/ml</td>
<td>62 15</td>
<td>68 36</td>
<td>120 54</td>
</tr>
<tr>
<td>Androstenedione, ng/ml</td>
<td>45a 12</td>
<td>18b 4</td>
<td>22b 4</td>
</tr>
</tbody>
</table>

a,b,cMeans in a row with unlike superscripts differ (P < 0.05).

1Cows were infused with rGRF or rbST from 117 to 180 d postpartum. Measurements were made in follicular fluid from follicles collected on d 5 of an estrous cycle at slaughter.

2EA follicles have more estradiol than progesterone in follicular fluid (7, 5, and 13 EA follicles for control, rGRF-, and rbST-treated groups, respectively); EI follicles have more progesterone than estradiol in follicular fluid (24, 16, and 20 EI follicles for control, rGRF-, and rbST-treated groups, respectively).

(infusion, daily, biweekly) could explain the discrepancies. Several examples follow:

a) Negative energy balance (41, 70) and lactation (10) decrease follicle and CL development and steroidogenesis. In addition, rbST treatment prolongs negative energy balance in lactating cows (41, 49), but does not alter energy balance in heifers or nonlactating cows (41, 70). Consequently, the effect of rbST on ovarian function in lactating cows may be modified by energy balance status. The nonpregnant cows in the present study were in positive energy balance when ovaries were removed (3, 68). Although rbST treatment may have altered follicular and CL growth and function without the confounding effect of negative energy balance, the rbST-treated cows in our study had a lower positive energy balance compared with control or rGRF-treated cows (3). Thus, we cannot rule out the possibility that differences in positive energy balance may modify rGRF or rbST treatment effects on follicular and luteal growth and function.

b) A threshold level of ST may be required to alter the dominant follicle growth or function. For example, serum and intrafollicular ST concentrations were lower in rGRF- compared with rbST-treated cows in the present study, perhaps explaining why dominant follicle growth and function were not altered in rbST-treated cows. Although several reports indicate that administration of rGRF or rbST increases ST levels in serum (3, 21), intrafollicular concentrations of ST were not reported.

c) The absence of (64), or an increase (8, 15) in twinning in rbST-treated cows may depend on timing of PGF2α, injection relative to stage of a follicular wave and number of estrogen-active follicles, which were not reported in previous studies. For example, using PGF2α to induce CL regression during d 7 of the estrous cycle causes ovulation of the first-wave dominant follicle (54). In the present study, a high proportion of follicles were estrogen-active in rbST-treated cows on d 5 of the estrous cycle, which if induced to ovulate, may form two CL. In support of this speculation, the number of estrogen-active follicles is higher in cattle selected for twinning (12), and most of the rbST-treated cows in the present study that had two CL also had two or more estrogen-active follicles. Taken together, this implies that number of estrogen-active follicles on d 5 of the estrous cycle is a reliable marker for ovulation rate.

d) The duration of rbST treatment may differentially affect CL function as measured by progesterone concentration. For example, administration of rbST increases progesterone in serum during the first and second estrous cycles compared with controls (16, 37, 56). However, during the third and subsequent estrous cycles, the concentration of progesterone is similar, or lower in rbST-treated cows compared with control cows (16, 31, 56) as shown in the present study. Of potential practical importance, if rbST-treated cows do not become pregnant during the first two estrous cycles after initiation of rbST treatment, the possibility exists that cows will become anovulatory (15, 69) because small concentrations of progesterone reduce expression of estrus or cause cessation of ovulation (33, 36). Also, cessation of ovulation and reduced expression of estrus could explain increased calving intervals after rbST treatment (14, 43). In further support of an altering effect
Figure 1. Effect of recombinant bovine growth hormone-releasing factor (rGRF) or recombinant bST (rbST) on IGF binding proteins (IGFBP) in follicular fluid from estrogen-active follicles in Holstein cows. Cows were infused with rGRF or rbST from 117 to 180 d postpartum. Ovaries were collected on d 5 of an estrous cycle after 63 d of treatment. Follicular fluid (25 µg of protein) from each estrogen-active follicle was subjected to SDS-PAGE, transferred to Immobilon P membranes, and incubated with 125I-IGF-I. The intensity of each band was determined as described previously. Bars depict mean (±SEM) intensity (arbitrary units) for IGFBP-2 (34 kDa), IGFBP-3 (39.7 to 45.4 kDa), IGFBP-4 (26.5 kDa), and IGFBP-5 (30.2 to 30.8 kDa). Number of estrogen-active follicles for control, rGRF-, and rbST-treated cows were 7, 5, and 13, respectively. Bars with unlike letters (a, b) among treatments differ (P < 0.05).

of long-term high serum levels of ST, the number of cows in the present study that did not have a CL following a PGF2α injection was greater in cows treated with rGRF and rbST compared with controls.

From the present study, there is clear evidence that exogenous rbST modifies follicular dominance, but the mechanism is unclear. Without exogenous rGRF or rbST, a decline in serum FSH concentrations ends the selection process that forms a single dominant follicle in cows (44). With increased intrafollicular ST after exogenous rbST as shown in our study, ST may synergize with FSH to increase the number of estrogen-active follicles, especially since ST receptors are located on granulosa cells from different size follicles in dairy cows (34). Direct positive effects of rbST on granulosa cell estradiol production may increase the number of estrogen-active follicles, and, therefore, explain the diminished putative dominance effect of first-wave dominant follicle after rbST injections (31, 39). Although androstenedione concentrations were lower in some follicles from rGRF- or rbST-treated cows compared with controls in the present study, estradiol concentrations were not reduced. This finding suggests that androgens were synthesized and rapidly aromatized to estradiol, rather than implying an overall negative effect of rGRF or rbST on androgen production.

The largest EA follicles were smaller in rbST-treated cows compared with controls in the present study. Although the mechanism explaining this result is unknown, others report that ST treatment decreases bovine granulosa cell proliferation in vitro (63). In addition, rbST decreases LH pulse frequency (56), which is associated with dominant follicles of smaller size (55). Also, administration of rbST increased CL number, but reduced their size and function in the present study. As explained earlier, it is likely that this effect of rbST is a consequence of its positive action on stimulating growth of multiple estrogen-active follicles, coupled with timing of PGF2α injection to induce ovulation of multiple EA follicles. Moreover, because serum progesterone concentrations were lower in rbST-treated cows in the present study, long-term treatment with rbST may have decreased CL function, perhaps by reducing
the number of LH receptors (50) or down-regulating ST receptor mRNA (32) in luteal cells. Alternatively, small dominant follicles may result in smaller CL because fewer granulosa and theca cells result in fewer luteal cells which, in turn, may produce less progesterone.

Although IGF-I concentrations in follicular fluid were similar among treatments in the present study, intrafollicular amounts of IGFBP-2 and -3 were higher in EA follicles from rbST-treated cows compared with controls and with rGRF-treated cows. In support of our results, another study shows that rbST treatment of cows increases IGFBP-3 in FF from the largest and second largest follicles (65). However, in contrast to our results, IGFBP-2 was decreased in the two largest follicles (65). Whether follicles were EA or EI was not reported (65). We speculate that the increased amounts of IGFBP-3 in follicular fluid in our study may be due to higher circulating IGFBP in serum of rbST-treated cows compared with rGRF-treated or control cows, as previously reported (68) for the cows in our study and by others (65). However, the reason intrafollicular amounts of IGFBP-2 and -4 were higher in the rbST-treated compared with rGRF-treated or control cows in our study is unknown. Perhaps, the enhanced intrafollicular amounts of ST in the rbST-treated cows increased ovarian production of IGFBP-2 and -4. In support of locally stimulated IGFBP production, it is established that theca cells express mRNA encoding for IGFBP-2 and -4 (1). The mechanism of how enhanced intrafollicular amounts of IGFBP increase number of EA follicles is unclear. Although several studies show that IGFBP-2 or 3 inhibit IGF-I action (30, 60, 67), other studies report that IGFBP-3 (4, 9, 30) or coinfusion of IGF-I and IGF-I antibodies in vivo [which may have an effect similar to IGFBP (66)] potentiates IGF-I action. In addition, IGFBP-2 and -3 increase number of ST receptors (59). Thus, the high intrafollicular amounts of IGFBP-2 and 3, coupled with the high concentrations of ST, as shown for the rbST-treated cows in our study, may explain why rbST-treated cows had multiple EA follicles compared with controls.

The rbST-treated, but not the rGRF-treated cows in our study had higher serum insulin concentrations compared with controls (68). Because insulin enhances FSH-induced estradiol production by granulosa cells in vitro (23, 25, 60, 63), the higher concentration of insulin in rbST-treated cows may also explain why this treatment group had more EA follicles.

Growth hormone-releasing factor effects on ovarian function may be mediated by ST or IGF-I and its binding proteins. Thus, the most likely explanation for why administration of rGRF did not alter follicular hierarchy in the present study is because intrafollicular threshold levels of ST and IGFBP were not achieved. Alternatively, exogenous rGRF stimulates the release of four ST variants (7), whereas the rbST injected into cows mimics only one of the ST variants. Thus, differences in biological potencies of ST variants (13) could also explain why rGRF- and rbST-treated cows responded differently in the present study. Finally, the possibility exists that rGRF may inhibit ST action on ovaries (58).

In contrast to our findings, treatment with GRF analo-logs increases size of large follicles and progesterone concentration in medium-sized follicles in mature heifers (61). However, GRF did not alter follicular hierarchy in heifers (61), as we observed in cows in the present study. In rats, GRF increases estradiol and progesterone production by granulosa cells (46). Taken together, GRF effects may be species specific or depend on stage of follicular differentiation or both.

In summary, daily infusions with rGRF or rbST for 63 d beginning 117 d postpartum to primiparous Holstein cows result in the following: a) both rGRF and rbST increased ST and IGF-I, but did not alter LH, FSH, or estradiol concentrations in serum; b) treatment with rbST increased number of CL, but decreased CL weight and serum concentration of progesterone compared with controls; c) rbST increased: number of cows with more than five follicles (>5 mm), number of EA follicles, intrafollicular concentrations of ST in follicles >5 mm, and amount of IGFBP-2, -3, and -4 in EA follicles compared with controls or rGRF-treated cows; d) rbST decreased size of EA follicles and androstenedione in EI follicles; and e) treatment with rGRF did not alter follicle size or CL weight and function compared with controls or rbST-treated cows, despite increased ST in all follicles, and decreased intrafollicular concentrations of androstenedione in EA and EI follicles compared with controls.

These findings lead to the conclusion that long-term treatment with rbST modifies growth and function of the first-wave dominant follicle and corpus luteum in cattle. The reason administration of rGRF did not alter follicular development is probably because intrafollicular concentration of ST and IGFBP were lower in the rGRF-treated compared with rbST-treated cows. Although the mechanism is unknown, it is speculated that sustained high intrafollicular levels of ST modify dominance by preventing atresia of subordinate follicles during a follicular wave. Based on the present study, rbST may prevent atresia of estrogen-active follicles by increasing intrafollicular levels of IGFBP-2, -3, and -4, which potentiate IGF-I bioactivity (4, 9, 30, 66), increase ST receptor (59), and, in turn, stimulate development of multiple estrogen-active follicles.

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