Ovarian Expression of Insulin-Like Growth Factor-I (IGF-I), IGF Binding Proteins, and Growth Hormone (GH) Receptor in Heifers Actively Immunized Against GH-Releasing Factor*

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ABSTRACT

Active immunization against GRF at 6 months of age delays puberty in beef heifers. The objectives of the present study were to determine whether active immunization against GRF at an earlier age would affect normal onset of puberty and follicular growth and to determine whether these changes were related to alterations in ovarian insulin-like growth factor I (IGF-I) or IGF binding protein (IGFBP) messenger RNA (mRNA) levels. Heifers were immunized against human serum albumin (HSAi; n = 15) or against GRF conjugated to HSA (GRFi; n = 18) at 3 months of age. A third group of heifers was not immunized (CON; n = 16). Immunization against GRF delayed puberty beyond 13 months of age in 75% of treated heifers. Unilateral ovariectomy at 191 days of age revealed that the delay in puberty was associated with a reduction in the number of large (>7 mm in diameter) follicles. Large follicles were present in only 22% of GRFi heifers compared to 77% of HSAi heifers. The number of small (≤3 mm in diameter) and medium (4 to 6 mm in diameter) follicles was not affected by GRFi. The percentage of 1- to 3-mm follicles that were atretic was not different between HSAi (65%) and GRFi (62%) heifers. Unilateral ovariectomy had no effect on age at puberty. Immunization against GRF decreased (P < 0.01) concentrations of IGF-I in serum (23 ± 2 ng/ml compared to HSAi heifers [199 ± 11 ng/ml]). IGF-I levels in follicular fluid (FFL) of medium and small follicles were also decreased by GRFi from 82 ± 3 ng/ml in HSAi heifers to 48 ± 6 ng/ml (P < 0.01). Levels of IGFBP-3 (determined by ligand blot analysis) in serum and FFL of small follicles were decreased by GRFi (P < 0.01). In contrast, IGFBP-2 serum levels were increased from 422 ± 32 ng/ml in HSAi heifers to 657 ± 6 ng/ml (P < 0.05). Likewise, IGFBP-2 levels in FFL from small and medium follicles were increased from 785 ± 44 ng/ml to 962 ± 44 ng/ml (P < 0.05). Ligand blot analysis indicated that IGFBP-2 levels were lower in FFL from large vs. small follicles. The band intensities of IGFBP-4 and -5 were drastically reduced (>80%) while the decreases in IGFBP-2 and -3 were less marked (<50%). The decreased levels of IGFBP-5 in FFL from large follicles was not associated with an increase in proteolytic fragments detectable by immunoblot analysis. While mRNA transcripts for IGF-I, GH receptor, and IGFBP-3, -4, and -5 were readily detectable in ovarian tissue, GRF had no effect on ovarian levels of mRNA for each of these proteins. This suggests that the decrease in follicular development associated with GRF may be related to changes in circulating IGF-I and/or IGFBPs.

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The mechanisms that regulate normal follicular development and onset of puberty are not clearly defined. Nutritional stress delays cyclic ovarian function or puberty in several species (1, 2). In this situation, the normal positive relationship between GH and insulin-like growth factor-I (IGF-I) is uncoupled, i.e. serum GH increases while IGF-I decreases (3, 4). Studies have demonstrated that exogenous GH alters ovarian follicular growth (5–7); however, it remains unclear whether this is a direct effect of GH or an effect mediated by IGFs and/or other factors. Either alone or in synergy with gonadotropins, IGF-I influences ovarian steroidogenesis and mitogenesis in vitro (5, 6). Specific forms of IGF-I binding proteins (IGFBPs) have been shown to modulate the ability of IGF-I to potentiate gonadotropin action in ovarian granulosa cells (5). Messenger RNA (mRNA) for IGF-I, the type I IGF receptor, and IGFBPs have been identified in ovarian tissue from several species (5), suggesting a potential autocrine/paracrine mode of action for IGF-I in the ovary. However, the ability of endocrine factors such as GH to regulate ovarian synthesis of the IGFs and their binding proteins is not well defined.

We have previously used the model of active immunization against GRF to evaluate the in vivo effects of IGF-I, GH, and/or GRF on puberty in bovine females (8). Immunization of heifers at 6 months of age decreased serum GH and IGF-I and delayed puberty 6 months beyond the typical age of 12 months.
Iifers were immunized against GRF at approximately 3 months of age. To determine whether local synthesis of IGF-I and IGFBPs was affected by GRF immunization, heifers were unilaterally ovariectomized (ULO) at 6 months of age and ovarian mRNA levels for IGF-I, IGFBP-2 through -5, and GH receptor were determined.

Materials and Methods

Peripheral bovine heifers were immunized against 1.5 mg GRF con-

Hybridization/ribonuclease protection assays using the Ambion (Austin, TX) kit as described by the manufacturer. Ten micrograms of RNA were hybridized with a 460-bp 32P-labeled bovine GH riboprobe, an 86-bp 32P-labeled IGF-I riboprobe corresponding to the A domain, or a 137-bp 32P-labeled β-actin riboprobe (14). The riboprobes were purified over Sephadex G50 columns, extracted with phenol/chloroform, and resuspended in nuclease protection buffer. The protected hybrids were separated on 8% acrylamide (Acrylamid; Promega, Madison, WI) denaturing gels. The relative intensities of protected RNA bands were quantified by PhosphorImager analysis and normalized for β-actin content.

Ligand and immunoblotting

The forms of IGFBP present in serum and FFL were visualized by

### Statistical analyses

Data were analyzed using the general linear models procedure of SAS (20). Split-plot ANOVA (21) was used for variables with multiple observations per heifer. Only data from small (≤3 mm in diameter) or medium (4 to 6 mm in diameter) follicles were included in the analyses of FFL IGF-I and IGFBP-2 since GRF induced an inadequate number of large (≥7 mm) follicles (n = 4) relative to untreated bovine heifers. Class of follicle (i.e., small or medium) was included in the initial analyses but subsequently deleted since neither class nor diameter contributed to variation in FFL IGF-I or IGFBP-2. Treatment was tested using the heifer within treatment × breed mean square as the error term. Episodes of LH release were defined using the method of Goodman and Karsch (22).

### Results

Active immunization against GRF at 3 months of age delayed puberty in more than 75% of heifers (P < 0.01; Fig. 1). Age at puberty was similar between HSAI and untreated heifers.
control heifers (Fig. 1), indicating that ULO had no effect on onset of puberty. GRFi was accompanied by a decrease in weight gain; weight (kilograms per day of age) at 383 days was greater in untreated control and HSAi (0.89 ± 0.02) than in GRFi (0.78 ± 0.02) heifers.

Serum concentrations of LH and frequency of pulsatile LH release were determined at 288 and 315 days of age (Table 1). Two heifers (CON and HSAi) became pubertal during this time and were omitted from LH analyses. Serum concentrations and frequency of LH release were similar in CON and HSAi heifers; thus, data were pooled and represented as HSAi. At 315, but not 288 days of age, serum LH was greater (P < 0.05) in GRFi than in HSAi heifers. Frequency of LH release was not affected by treatment; however, across treatment, frequency increased with age (288 vs. 316 days, P < 0.05).

The number of small, medium, or large surface ovarian follicles in GRFi or HSAi heifers at 191 days of age is presented in Fig. 2. The number of large follicles was drastically decreased (P < 0.05) by GRFi. Only 22% of GRFi heifers had follicles ≥ 7 mm compared to 77% of HSAi heifers. In contrast, the number of small and medium follicles was not affected by treatment. The incidence of atresia was determined in small follicles between 1 and 3 mm in diameter. The percentage of follicles that were atretic was not affected by GRFi (Table 2).

Concentrations of IGF-I and IGFBP-2 in serum and FFL from follicles ≤ 6 mm at the time of ULO are presented in Fig. 3. Only FFL from follicles ≤ 6 mm was used for analysis because of a limited number of large follicles in GRFi heifers (n = 4). Neither class (small vs. medium) nor diameter contributed to variation in IGF-I or IGFBP-2; thus data were pooled for presentation. Serum levels of IGF-I were decreased from 109 ± 11 ng/ml in HSAi heifers to 23 ± 2 ng/ml in GRFi heifers (P < 0.01). Likewise, levels of IGF-I in FFL were lower (P < 0.01) in GRFi heifers (48 ± 6 ng/ml) compared to HSAi heifers (82 ± 3 ng/ml). In contrast, heifers immunized against GRF had higher circulating levels of IGFBP-2 compared to HSAi heifers (657 ± 6 ng/ml vs. 422 ± 32 ng/ml; P < 0.05). This treatment effect was also observed with FFL IGFBP-2 levels (926 ± 44 ng/ml in GRFi heifers vs. 785 ± 44 ng/ml in HSAi heifers; P < 0.05).

Ligand blot analysis of serum from HSAi and GRFi heifers is shown in Fig. 4. The intensities of the IGFBP-3 bands in serum were less (P < 0.01) in GRFi (1980 ± 276) than in HSAi (3770 ± 455) heifers. Likewise, the intensities of the IGFBP-4 bands were decreased 30% by GRFi (590 ± 66 vs. 413 ± 41; P < 0.05). GRFi had no effect on the intensities of the doublet bands migrating at 29,000–31,000 (possibly IGFBP-5). While serum IGFBP-2 levels determined by RIA were increased 55% in GRFi heifers, a significant increase was not observed when IGFBP-2 levels were quantitated by ligand blot analysis. To ensure that the higher values obtained by RIA were not due to an increase in proteolytic fragments that were detected in the RIA, the Western blots were immunoblotted with antisera against IGFBP-2, which detects IGFBP-2 proteolytic fragments (23). While IGFBP-2 fragments of 22 and

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**TABLE 1.** Serum LH and pulse frequency in heifers actively immunized against GRF, HSA, or nonimmunized (CON)

<table>
<thead>
<tr>
<th>288 days of age</th>
<th>HSAi*</th>
<th>GRFi</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum LH (ng/ml)</td>
<td>0.5</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Pulse frequency (peaks/8 h)</td>
<td>0.86</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>315 days of age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum LH (ng/ml)</td>
<td>0.7</td>
<td>0.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Pulse frequency (peaks/8 h)</td>
<td>1.6</td>
<td>1.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 22 heifers, 33 samples/heifer). <sup>a</sup> Control and HSAi were similar; therefore data were pooled. <sup>b</sup> P < 0.05 compared with GRFi.
of HSAi animals indicated that total IGFBP binding activity was lower in FFL from large vs. small follicles (Fig. 6, panel A). IGFBP-4 and IGFBP-5 levels were dramatically reduced (86 ± 9% and 88 ± 9%, respectively; n = 4) in FFL from large follicles. In contrast, smaller decreases in IGFBP-2 (38 ± 17%) and IGFBP-3 (29 ± 11%) were observed. While IGFBP-5 fragments of 21–22 kDa were detectable in FFL by immunoblot analysis, the intensities of these bands corresponded with the intact IGFBP-5 bands (Fig. 6, panel B).

Transcripts for IGFBP-2, -3, -4, and -5 mRNAs were readily detectable by Northern analysis in ovarian tissue of both GRFi and HSAi heifers (Fig. 7). However, levels of mRNA for each of the IGFBPs were not affected by treatment. Similarly, ovarian levels of IGF-I and GH receptor mRNA (analyzed by

Fig. 3. Concentrations (mean ± SEM) of IGF-I (panel A) or IGFBP-2 (panel B) in serum or FFL collected at time of ULO (191 days of age). Heifers were actively immunized against GRF or HSA at 94 days of age. Data were pooled from follicles with diameters ≤ 6 mm. Serum and FFL IGF-I were decreased by GRFi. In contrast, serum and FFL concentrations of IGFBP-2 were increased by GRFi. Across treatments, concentrations of IGFBP-2 were greater in FFL than in serum.

Fig. 4. Forms of IGFBPs present in serum at the time of ULO (day 191). Heifers were actively immunized against GRF (G) or HSA (H) at 94 days of age. Serum (1.5 µl) was subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and probed with [125I]IGF-I. Molecular weight standards were run in parallel. Immunoblot analysis identified bands as IGFBP-3 (M, 46,000 and 43,000), IGFBP-2 (M, 34,000), and IGFBP-4 (24,000).

Fig. 5. Forms of IGFBPs present in FFL from follicles ≤ 3 mm in diameter at the time of ULO (day 191). Heifers were actively immunized against GRF (G) or HSA (H) at 94 days of age. FFL (1.5 µl) was subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and probed with [125I]IGF-I. Molecular weight standards were run in parallel. Immunoblot analysis identified bands as IGFBP-3 (M, 46,000 and 43,000), IGFBP-2 (M, 34,000), and IGFBP-4 (24,000).

Fig. 6. Forms of IGFBPs present in FFL from small (S) follicles ≤ 3 mm in diameter or large (L) follicles ≥ 7 mm in diameter at the time of ULO (day 191). Heifers were actively immunized against HSA at 94 days of age. Lanes 1–2, 3–4, and 5–6 represent a direct comparison of FFL from small vs. large follicles for each of three animals. FFL (1.5 µl) was subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and probed with [125I]IGF-I (A) or antisera-specific for IGFBP-5 (B) as described in Materials and Methods. Molecular weight standards were run in parallel.
Fig. 7. Northern blots of total RNA (20 μg) isolated from ovaries of heifers actively immunized against GRF (G) or HSA (H) at 94 days of age. Heifers were ULO at 191 days of age. RNA was hybridized with complementary DNA probes for bovine IGFBP-2 and -3 and human IGFBP-4 and -5. GRFi had no effect on levels of any of the IGFBPs.

ribonuclease protection assay) were not different between GRFi and HSAi heifers (Fig. 8).

Discussion

These results confirm our previous finding that immunoneutralization of GRF delays puberty in bovine females (8). Furthermore, a greater percentage of heifers (75% vs. 50%) failed to reach puberty by 12 months of age when immunization against GRF was initiated at 3 months of age compared to 6 months of age in our previous study. This indicates that follicular growth between 3 and 6 months of age may be critical to normal onset of puberty in heifers. In the present study, the number of large follicles was decreased by GRFi, while the number of small and medium follicles was not affected. This contrasts with the study of Gong et al. (7) in which treatment of postpubertal heifers with exogenous GH increased the number of small follicles but not the number of large follicles at each follicular wave. Hence GH may influence follicular development differently in pre- vs. postpubertal animals. Our data suggest that the GRF/GH axis is involved in the dynamics of follicular growth as early as 6 months in heifers. This is particularly important since the majority of studies aimed at identifying endocrine changes associated with puberty have focused on the 1- to 2-month period preceding puberty.

Mechanisms by which immunoneutralization of GRF alters follicular growth have not been clearly defined. Immunization against GRF at 3 months of age decreased concentrations of IGF-I in both serum and FFL. The ability of IGF-I to stimulate granulosa cell mitogenesis in vitro and to amplify gonadotropin action on steroidogenesis in granulosa and thecal cells suggests that it may be an important regulator of folliculogenesis (5). The role of local vs. systemic IGF-I in mediating these effects is unclear. IGF-I mRNA transcripts have been detected in rat and human ovarian tissue (24, 25), and bovine and porcine granulosa cells have been shown to synthesize IGF-I (26, 27). In the present study the abundance of IGF-I transcripts in whole ovarian tissue was not affected by GRFi, suggesting that local synthesis of IGF-I was not altered. However, ovarian IGF-I availability may have been decreased via an endocrine mechanism since serum IGF-I levels were drastically reduced in GRFi heifers. Few studies have examined the hormonal regulation of ovarian IGF-I levels in vivo. In the immature hypophysectomized rat model, GH increased IGF-I mRNA levels in the liver but not the ovary, while estrogen appeared to be the pivotal inducer of ovarian IGF-I expression (28). However, a recent study reported that ovarian levels of IGF-I mRNA were decreased in young rats made GH deficient by treatment with monosodium glutamate. This effect was reversed by GH treatment (29). Similar to our findings, Samaras et al. (30) reported that ovarian IGF-I mRNA levels were not altered in prepubertal gilts after 20 or 40 days of exogenous GH treatment in spite of increased levels of IGF-I in both serum and FFL. These studies suggest that GRF and/or GH may not regulate ovarian synthesis of IGF-I in domestic species.

Ovarian granulosa and thecal cells secrete multiple forms of IGFBP in vitro (31–34), and several forms have been identified in FFL (15, 31, 35, 36). Therefore, the ability of IGF-I to influence follicular development may be largely regulated by its interaction with the IGFBPs. Exogenous addition of IGFBP-1 through -5 has been shown to inhibit FSH-stimulated cAMP generation, steroidogenesis, and mitogenesis of cultured granulosa cells (34, 37–40). These data suggest a role for the IGFBPs as antagonogtropins; by sequestering IGF-I they may negate its ability to amplify gonadotropin action. In the present study levels of IGFBP-2 in FFL from small follicles were increased by GRFi while IGFBP-3 levels were decreased. Recently Grimes et al. (41) reported a positive correlation between FFL IGFBP-3 levels and follicle diameter in the porcine ovary (41). In contrast, IGFBP-2 was found to be related more to atresia than to follicle size, suggesting specific roles for individual forms of IGFBP. Given the elevated levels of IGFBP-2 observed in FFL from GRFi heifers,
Recent studies have suggested that IGFBP-3 produced by vascular elements may be involved in luteolysis of the corpus luteum in the rat (51). IGFBP-3 was detected only in the endothelium of ovarian blood vessels in the human ovary (48). Our design did not allow delineation of cell types producing IGFBP; however, luteal tissue can be ruled out as a source since heifers were prepubertal.

A direct effect of GH on the ovary is possible since GH receptors have been identified in ovarian tissue. GH has been reported to enhance mitogenesis and steroidogenesis in granulosa cells in vitro (52, 53). In addition, gonadotropin-stimulated differentiation of rat granulosa cells was augmented by GH (53). However, levels of GH receptor mRNA were not altered by GRF in the present study, suggesting that GRF does not inhibit ovarian function via a decrease in GH receptor gene expression.

It is interesting that mean serum LH was greater in GRF than in HSA heifers at 315 days of age. This is an age at which LH might be expected to increase in association with impending puberty, yet only 25% of GRF heifers were pubertal by 14 months of age. A similar elevation of serum LH has been reported in mature cyclic GRF beef cows (53) and in 5- to 6-month-old GRF heifers (54). It is possible that GRF alters LH sensitivity to estradiol since Schoppee et al. (55) found that GRF heifers had elevated LH after exogenous estradiol.

In conclusion, active immunization against GRF delayed puberty beyond 14 months of age in bovine females by altering follicular growth between 3 and 6 months of age. Treatment was also associated with decreased concentrations of IGF-I and IGFBP-3 and increased IGFBP-2 in FFL. However, ovarian expression of IGF-I and IGFBP-2, -3, -4, or -5 was not affected, suggesting that the changes in systemic levels of IGF-I and IGFBPs that occurred during GRF immunization may play a role in inhibiting follicular growth.

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