Effect of Somatotropin and/or Equine Chorionic Gonadotropin on Serum and Follicular Insulin-Like Growth Factor I and Insulin-Like Growth Factor Binding Proteins in Cattle[1]

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ABSTRACT

We investigated the effect of administration of somatotropin (ST) and/or eCG on insulin-like growth factor I (IGF-I) and IGF-binding proteins (IGFBP) in serum and follicular fluid (FFL) of cattle actively immunized against growth hormone-releasing factor (GRF). Cyclic beef cattle, previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH$_2$ conjugated to human serum albumin (synthesized and provided by Hoffmann-LaRoche, Inc., Nutley, NJ; GRFi, n = 31) or to human serum albumin alone (HSAi, n = 26), received (i.m.): 1) 25 mg recombinantly derived methionyl somatotropin (rbST, n = 14; sometribove provided by Monsanto Co., St. Louis, MO); 2) 1100 IU eCG (n = 10); 3) rbST and eCG (rbST-eCG, n = 15); or 4) vehicle (VEH, n = 17) at 0 and 24 h after receiving prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). Serum samples were collected at 0 and 40 h after PGF$_{2\alpha}$, and the ovary bearing the largest follicle (DOM) was removed 44.0 ± 0.5 h after PGF$_{2\alpha}$; FFL was harvested from DOM and the subordinate follicle (SUB). Before treatment (0 h), GRFi cows had lower serum ST (0.6 ± 0.2 vs. 2.2 ± 0.2 ng/ml; $p < 0.01$) and IGF-I (26 ± 4 vs. 72 ± 4 ng/ml; $p < 0.01$), but greater IGFBP-2 (594 ± 48 vs. 384 ± 52 ng/ml; $p < 0.01$) than HSAi cows. Serum and FFL concentrations of IGF-I or IGFBP-2 were not different between rbST- and rbST-eCG-treated cows or between VEH- and eCG-treated cows at Hour 40 after the initial treatment injection; therefore, data were combined and designated as rbST and VEH, respectively. Serum IGF-I was increased to a greater extent (percentage increase above 0 h) by rbST treatment in GRFi (362 ± 24) than in HSAi (176 ± 16) cows (immunization by treatment, $p < 0.01$). Across GRFi and HSAi, rbST lowered serum IGFBP-2 (342 ± 31 vs. 541 ± 27 ng/ml, rbST vs. VEH; $p < 0.01$). Diameters of DOM or SUB were not affected by immunization or treatment. Concentrations of IGF-I and IGFBP-3 (determined by ligand blot analysis) in FFL from both DOM and SUB were lower ($p < 0.05$) in GRFi than in HSAi cows. In contrast, IGFBP-2 in FFL was elevated in SUB, but not DOM, in GRFi compared to HSAi cows. Ligand blot analyses indicated that IGFBP-4 and IGFBP-5 were markedly higher in FFL from SUB in GRFi than in HSAi cows, but not different for DOM. Administration of rbST increased IGF-I and decreased IGFBP-2 in DOM and SUB. In conclusion, GRFi decreased serum and FFL concentrations of IGF-I, while it increased concentrations of IGFBP-2 in serum and in FFL from SUB, but not DOM. Treatment with rbST increased serum and FFL IGF-I, but decreased both serum and FFL IGFBP-2 (in both DOM and SUB). The specific roles that IGF-I and IGFBP play in folliculogenesis are yet to be determined; of particular interest is the divergent effect of GRFi on IGFBP in dominant vs. subordinate follicles.

INTRODUCTION

Somatotropin (ST) is a key regulator of somatic growth. In ruminants, hypophysectomy [1] or active immunization against growth hormone-releasing factor (GRF) [2–4] decreases body weight gain and skeletal growth, while treatment with exogenous ST enhances deposition of lean tissue [5]. A portion of the effects of ST are mediated by insulin-like growth factors (IGF). The IGF circulate bound to specific binding proteins (IGFBP), which modulate their function [6].

In vitro studies have shown that IGF-I enhances FSH-stimulated steroid production in murine [7] and porcine granulosa cells [8,9]. Schams [10] has shown that IGF-I increases, in a dose-dependent manner, the production of

progesterone (P$_4$) and oxytocin to values greater than those obtained with FSH alone in bovine granulosa cells. Furthermore, gonadotropins increase IGF-I secretion by porcine granulosa cells [11]. IGFBP have been shown to inhibit steroid production by granulosa cells in vitro [12] and to inhibit ovulation in vivo [13]. These observations support a possible role for ST, IGF-I, and/or IGFBP in the regulation of ovarian function, specifically folliculogenesis.

In the presence of insulin, ST has been shown to enhance cell growth in bovine in vitro-cultured granulosa cells [14] and P$_4$ production in bovine [14] and porcine [15] in vitro-cultured granulosa cells. Jia et al. [16] have demonstrated that ST augments gonadotropin-stimulated differentiation of in vitro-cultured granulosa cells obtained from immature rats. In vivo effects of ST on ovarian function have been studied in cattle [4, 17–21] and prepubertal gilts [22–25], but the results have been variable. Active immunization against GRF has been shown to decrease serum ST and IGF-I and to delay puberty in beef heifers [3, 4]. Heifers immunized against GRF that failed to reach puberty by 18.5 mo of age had lower serum IGF-I concentrations at the time of primary GRF immunization (6 mo of age). We admin-

istered ST and/or eCG to beef cows that had been previously immunized against GRF to clarify the interactions between ST, IGF-I, IGFBP, and gonadotropins at the level of the ovary. Specifically, our objective was to quantify IGF-I and IGFBP in follicular fluid (FFL) from cyclic cows with elevated, normal, or low serum ST and IGF-I.

MATERIALS AND METHODS

Cyclic nulliparous and primiparous Angus and Charolais (n = 43) [3, 26] or multiparous Angus and Hereford (n = 12) females [27], previously immunized against GRF or human serum albumin, were used. The 2- to 3-yr-old Angus and Charolais cows weighed 407 ± 11 and 440 ± 14 kg, respectively. The multiparous Angus and Hereford cows were 5–6 yr of age and weighed 475 ± 31 kg. Cows were immunized with 1.5 mg GRF-(1–29)-Gly-Gly-Cys-NH$_2$ conjugated to 1.5 mg human serum albumin (GRFi; Hoffmann La-Roche, Inc., Nutley, NJ) or with 1.5 mg human serum albumin (HSAi; Sigma Chemical Co., St. Louis, MO) at 6 mo of age [3, 26] or at 3–4 yr of age [27]. Cows were administered booster immunizations as previously described [3, 26, 27]; however, the most recent booster immunization would have been given at least 3 mo before initiation of this study. A final booster injection was given 4 wk before the study began.

Estrous cycles were synchronized by three i.m. injections of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; Lutalyse, Upjohn, Kalamazoo, MI) given 14 days apart (1000–1400 h). Prior to synchronization, cows were maintained on grass-legume pasture. At the time of the first PGF$_{2\alpha}$ injection, cows were moved to open-sided pens with slotted floors (30 m × 9 m). Cows were fed a diet of sorghum silage and concentrate calculated to provide 100% of NRC recommendations [28] for a 500-kg mature, nonlactating beef cow. Cows were divided randomly within breed (Angus, Charolais, or Hereford) and immunization status (GRFi, n = 31, 398 ± 18 kg; or HSAi, n = 26, 443 ± 9 kg) into one of four treatment groups: 1) rbST: i.m. injection (25 mg) of rbST (sometribove; Monsanto Co., St. Louis, MO) (GRFi-rbST, n = 10; HSAi-rbST, n = 4); 2) eCG: i.m. injection (1100 IU) of eCG (Diosynth Inc., Chicago, IL) (GRFi-eCG, n = 5; HSAi-eCG, n = 6); 3) rbST-eCG: i.m. injections of rbST (25 mg) and eCG (1100 IU) (GRFi-rbST-eCG, n = 6; HSAi-rbST-eCG, n = 9); or 4) VEH: i.m. injection (2.5 ml) of sterile 35 mM NaHCO$_3$ (Sigma) (GRFi-VEH, n = 10; HSAi-VEH, n = 7). We ensured that 4–6 cows were in each treatment; excess animals were allocated to rbST and VEH treatments. The dosage of eCG specified above was used to achieve follicular stimulation without superovulation [29]. Cows received their respective treatments at 0 and 24 h from the last injection of PGF$_{2\alpha}$. Procedures were approved by the North Carolina State University Animal Care and Use Committee.

Approximately 44.0 (± 0.5) h after the last injection of PGF$_{2\alpha}$, ovaries were examined via the rectum by real-time

ultrasound (Aloka 500V; Corometrics Medical Systems, Inc., Wallingford, CT) using a 7.5-MHz transducer (Aloka, UST-660–7.5; Corometrics). Time after PGF$_{2\alpha}$ was based on a preliminary experiment in which ovaries were removed at 24 or 48 h after PGF$_{2\alpha}$ administration. Epidural anesthesia was produced by infiltrating the epidural space between the last sacral and first caudal vertebrae with 5 ml of 2% lidocaine HCl (Lidocaine; Sigma-Aldrich Corp., Milwaukee, WI). Once identified, the ovary bearing the largest follicle was unilaterally removed by inserting an écraseur through an incision in the dorsal wall of the vagina. Cows that had ovulated were excluded. Ovaries were placed on ice and transported to the laboratory. Diameters of the largest (DOM) and subordinate follicle (SUB), on the single ovary removed, were recorded; DOM was dissected free of ovarian stroma, and FFL was aspirated from DOM and SUB. FFL was centrifuged at 1500 × g for 15 min to remove cellular debris and then aspirated and stored at −20°C until analyzed for IGF-I and IGFBP. All steps were completed within 30 min of ovariectomy.

Blood samples were collected via jugular or coccygeal venipuncture three times per week during estrous synchronization to monitor P$_4$ concentrations. Single blood samples were also collected before initiation of treatment (0 h) and before ovariectomy (40 h). Blood samples were stored at 4°C for at least 8 h and centrifuged at 1500 × g for 30 min; serum was stored at −20°C until analyzed for ST, IGF-I, and IGFBP.

Assays

Serum concentrations of P$_4$ were determined using P$_4$ (P-0130, Sigma) standards prepared in 0.01 M PBS-0.1% gelatin (G8–500; Fisher Scientific, Atlanta, GA) by a solid-phase RIA (Diagnostic Products Corp., Los Angeles, CA). Average intra- and interassay coefficients of variation (CV) were 6.6 and 10.1%, respectively, for five assays. Sensitivity, defined as 90% of total binding, was 0.5 ng/ml.

Serum concentrations of ST were determined in a single assay by the procedure described by Armstrong and Spears [30]. Intraassay CV and sensitivity, as described previously, were 9.6% and 0.6 ng/ml, respectively. Serum and FFL concentrations of IGF-I were determined as described by Houseknecht et al. [31] with modifications [32, 33]. Addition of 0.5, 1, and 4 ng of IGF-I (before extraction) to FFL yielded recoveries of 79, 81, and 83%. Assay of 5, 10, 20, and 40 μl of serum and FFL resulted in a line parallel to that for known concentrations of IGF-I. Average intra- and interassay CVs were 8.3 and 10.2%, respectively, for four assays. Assay sensitivity, as described previously, was 6.2 ng/ml.

Concentrations of IGFBP-2 in serum and FFL were determined by double-antibody RIA as detailed by Cohick et al. [34]. Bovine IGFBP-2 was purified from Madin-Darby bovine kidney cell-conditioned medium and used for iodination, standards, and generation of specific IGFBP-2 antisera. Serum and FFL samples were diluted 1:20 with RIA
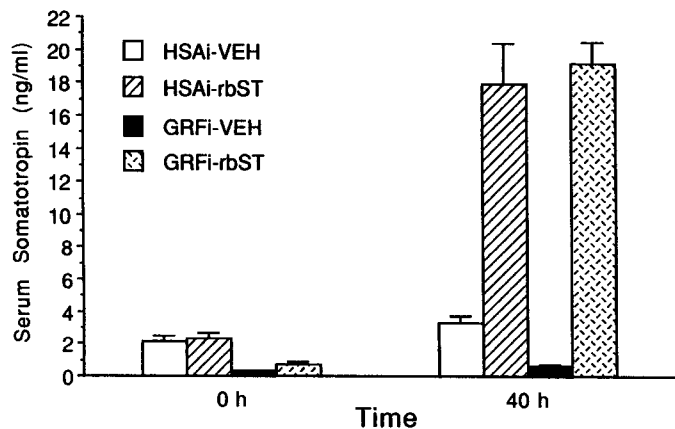
FIG. 1. Concentrations of ST (mean ± SEM) at 0 and 40 h after treatment with rbST, eCG, rbST and eCG (rbST-eCG), or vehicle in cyclic cows previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH$_2$-human serum albumin (GRFi, n = 26) or human serum albumin (HSAi, n = 24). Treatments were administered (i.m.) at 0 and 24 h from injection of PGF$_{2\alpha}$. Concentrations of ST were similar in rbST and rbST-eCG cows; thus data were combined and presented as rbST (n = 25). ST was also similar in vehicle- and eCG-treated cows and data were combined for presentation (presented as VEH, n = 25). The treatment-by-immunization interaction was not significant. Serum ST was lower (p < 0.01) in GRFi than in HSAi cows at Hour 0, but not at Hour 40. Concentrations of ST were increased (p < 0.01) at 40 h after rbST treatment.

buffer, and 5 µl was analyzed. The sensitivity of the assay was 0.6 ng/ml. Average intra- and interassay CVs were 6.7 and 8.9%, respectively, for two assays.

Ligand and Immunoblotting

Serum and FFL IGFBP from individual GRFi or HSAi cows treated with VEH were visualized by ligand blotting. Procedures were carried out as described by Hossenlopp et al. [35] with modifications as follows: aliquots of serum and FFL (10 µl) were mixed with 130 µl of sample buffer (single-strength), and 14 µl of diluted sample was loaded onto 12.5% discontinuous SDS-polyacrylamide gels. Gel electrophoresis was performed with a Mighty Small II Apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at 25 mA per gel until the dye front reached the bottom of the gel. Proteins were then transferred to polyvinyldifluoride (PVDF) filters (Immobilon P; 0.45-µm pore size; Millipore Co., Bedford, MA) with a semi-dry electroblotter (Janssen Life Sciences Products, Piscataway, NJ) at 70 mA for 75 min. The filters were probed overnight with [^{125}I]IGF-I, washed, and audioradiographed by exposure to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY). Molecular weight estimates were based on prestained protein standards (Amersham Corp., Arlington Heights, IL). Band intensities were determined by use of the Hoeffer 350 program and densitometer.

To identify specific forms of IGFBPs, immunoblotting of the filters was performed with antibodies against bovine IGFBP-2 and -3 [34] and human IGFBP-5 [36]. Primary antibodies were diluted in TBS (200 mM NaCl, 50 mM Tris,

pH 7.4) containing 1% BSA and used at dilutions of 1:2000 (IGFBP-2) and 1:1000 (IGFBP-3 and -5). The filters were rinsed for 15 min in TBS containing 5% Tween-20, blocked for 15 min (IGFBP-2 and -3) or 2 h (IGFBP-5) with TBS containing 3% BSA, and incubated overnight at room temperature with the primary antibody. The filters probed with IGFBP-2 or -3 antisera were washed with TBS containing 0.1% NP-40 and 0.03% Triton X-100 (three times for 10 min each) and then incubated for 3 h with alkaline phosphatase-conjugated second antibody (Sigma). After washing with TBS containing 0.1% NP-40 and 0.03% Triton X-100 (three times for 10 min each), alkaline phosphatase activity was detected via the Problot immunoblotting system (Promega, Madison, WI). After the primary antibody incubations, the filters probed with IGFBP-5 antisera were washed in TBS containing 0.1% Tween-20 (twice for 15 min each), then washed for 15 min in TBS containing 1.0% Tween-20, and again washed in TBS containing 0.1% Tween-20 (twice for 15 min each); they were incubated for 3 h with peroxidase-conjugated anti-guinea pig IgG (Sigma). The washes in TBS containing Tween-20 were repeated as described above, and peroxidase activity was detected by means of the ECL chemiluminescent detection system (Amersham).

Statistical Analyses

All analyses were conducted by means of GLM procedures [37]. Hormone data from serum collected at 0 and 40 h were analyzed using a model containing immunization (GRFi vs. HSAi), treatment (VEH, rbST, ECG, rbST-ECG), immunization by treatment, cow within immunization by treatment, hour, immunization by hour, treatment by hour, and immunization by treatment by hour. The effects of immunization, treatment, and immunization by treatment were tested using cow within immunization by treatment mean square as the error term. Initial analyses revealed that neither breed nor interactions involving breed were significant; thus effects involving breed were omitted from subsequent analyses. Due to the pretreatment difference in serum IGF-I between GRFi and HSAi, serum IGF-I concentrations at 40 h were expressed as percentage of pretreatment serum IGF-I. Treatment means were compared by Student Newman-Keuls test using cow within immunization by treatment as the error term.

Hormone concentrations from FFL (IGF-I and IGFBP-2) were analyzed through use of a model containing immunization, treatment, and immunization by treatment. Initial analyses revealed that neither breed nor interactions involving breed were significant; thus effects involving breed were omitted from subsequent analyses. Diameter, diameter by treatment, and diameter by immunization were included as covariates; covariates not contributing to variation (p > 0.1) were deleted from subsequent analyses. Treatment means were compared by Student Newman-Keuls test for ST, IGF-I, and IGFBP-2. A separate analysis was conducted to determine the effect of source (DOM, SUB, or
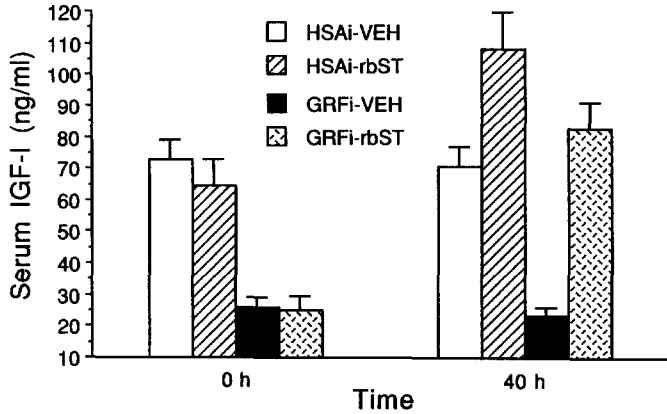
FIG. 2. Serum concentrations of IGF-I (mean ± SEM) at 0 and 40 h after treatment with rbST, eCG, rbST and eCG, or vehicle in cyclic cows previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH₂-human serum albumin (GRFi, n = 26) or human serum albumin (HSAi, n = 24). Treatments were administered (i.m.) at 0 and 24 h from administration of PGF₂α. Concentrations of IGF-I were similar in rbST and rbST-eCG cows; thus data were combined and presented as rbST (n = 25). Serum IGF-I was also similar in vehicle- and eCG-treated cows and data were combined for presentation (as VEH, n = 25). Serum IGF-I was greater (p < 0.01) in HSAi than in GRFi at 0 h. At 40 h, rbST increased serum IGF-I to a similar level in GRFi and HSAi cows.

serum) on concentrations of IGFBP-2. The model used for analysis was similar to the model used for serum hormone analyses, with hour replaced by source. Pearson correlation coefficients were calculated across treatment and immunization groups between follicular diameter and FFL concentrations of IGF-I or IGFBP-2. Band intensities of IGFBP other than IGFBP-2 were analyzed by independent t-test.

TABLE 1. Mean diameter (mm) of the largest (DOM) and second largest follicles (SUB) removed 44 h after injection of PGF₂α from cyclic cows immunized against GRF[a] or HSA[b] and administered rbST, rbST-eCG, eCG, or vehicle.

Treatment[c]	Immunization	
	HSAi	GRFi
	DOM	
rbST	15.5 ± 1.7*	15.3 ± 2.0
rbST-PMSG	17.0 ± 1.6	15.6 ± 1.9
PMSG	13.6 ± 2.1	11.0 ± 3.0
VEH	15.7 ± 0.8	18.6 ± 2.2
	SUB	
rbST	9.8 ± 2.4	7.3 ± 1.2
rbST-PMSG	10.6 ± 1.4	10.8 ± 1.6
PMSG	8.8 ± 1.5	6.2 ± 1.6
VEH	9.1 ± 1.1	8.4 ± 1.0

[a]GRF-(1-29)-GLY-GLY-CYS-NH₂ conjugated to human serum albumin (GRFi).
[b]Human serum albumin (HSAi).
[c]Treatments were administered (i.m.) at 0 and 24 h after injection of PGF₂α.
*Mean ± SEM.

RESULTS

Seven cows ovulated before ovariectomy and were deleted from the study. Cows that had ovulated were distributed evenly across immunization and treatment groups.

Differences were not detected (p > 0.1) between rbST and rbST-eCG treatments in any of the serum or FFL variables measured; therefore, these treatments were combined for presentation (represented by rbST). The effect of treatment with eCG was similar (p > 0.1) to that of VEH
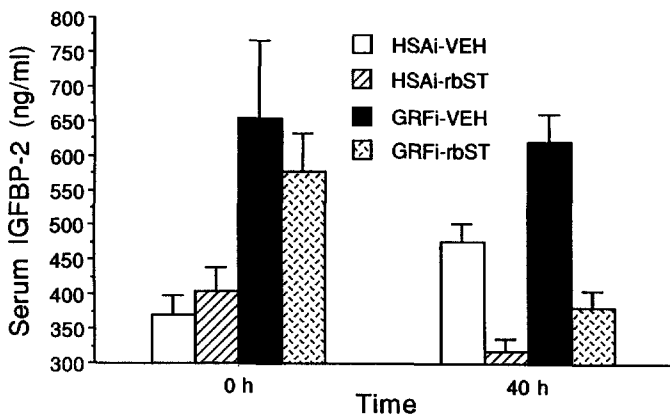
FIG. 3. Serum concentrations of IGFBP-2 (least-squares mean ± SEM) at 0 and 40 h after treatment with rbST, eCG, rbST and eCG, or vehicle in cyclic cows previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH₂-human serum albumin (GRFi, n = 26) or human serum albumin (HSAi, n = 24). Treatments were administered (i.m.) at 0 and 24 h from PGF₂α. Serum IGFBP-2 was similar in rbST- and rbST-eCG-treated cows; thus data were combined and presented as rbST (n = 25). Concentrations of serum IGFBP-2 were also similar in vehicle-and eCG-treated cows and data were combined (presented as VEH, n = 25). The treatment-by-immunization interaction was not significant. Serum IGFBP-2 was greater (p < 0.01) in GRFi than in HSAi cows at Hour 0, but not at Hour 40. Across immunization, concentrations of serum IGFBP-2 were lower (p < 0.01) in rbST than in VEH cows.
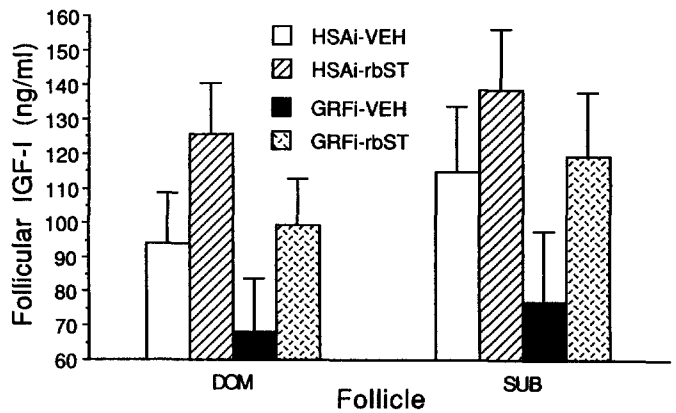
FIG. 4. Mean (± SEM) concentrations of IGF-I in the largest (DOM) and second largest (SUB) follicles at 44 h after treatment with rbST, eCG, rbST and ECG, or vehicle in cyclic cows previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH₂-human serum albumin (GRFi, n = 26) or human serum albumin (HSAi, n = 24). Treatments were administered (i.m.) at 0 and 24 h from injection of PGF₂α. All cows were unilaterally ovariectomized at 44 h. FFL concentrations of IGF-I were similar in rbST and rbST-eCG cows; thus data were combined and presented as rbST (n = 25). Similarly, IGF-I was not different in vehicle- and eCG-treated cows and data were combined (presented as VEH, n = 25). Concentrations of IGF-I in DOM and SUB were lower (p < 0.05) in GRFi than in HSAi cows. Across immunization, treatment with rbST increased (p < 0.01 and p < 0.07, respectively) IGF-I in DOM and SUB. The treatment-by-immunization interaction was not significant.
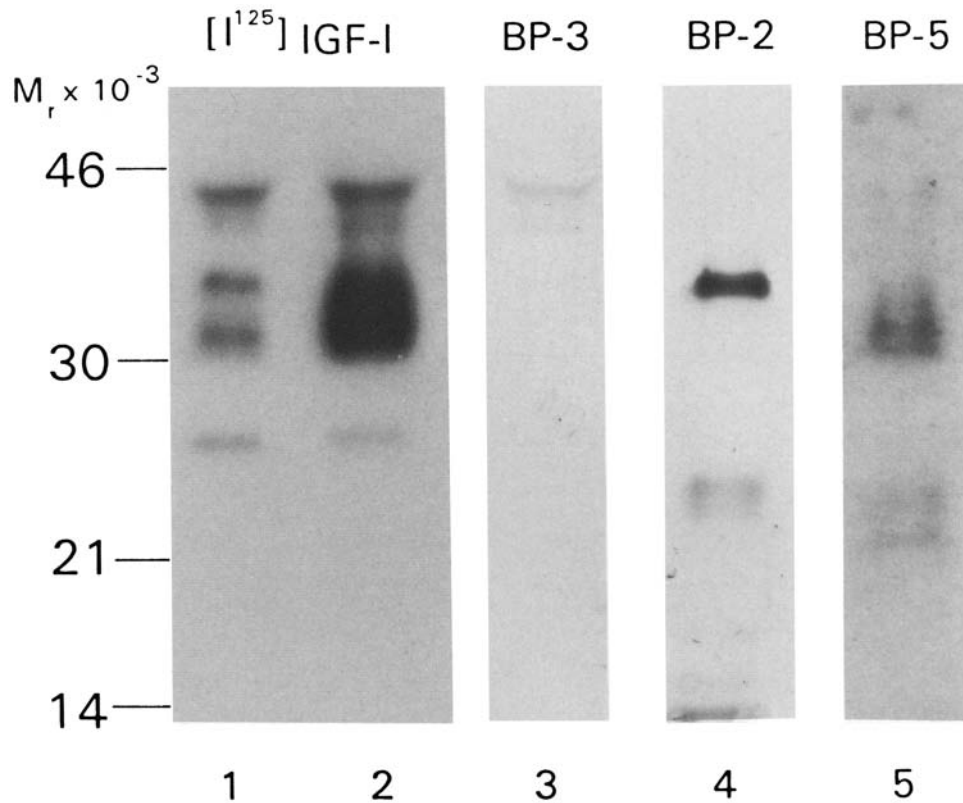
FIG. 5. Identification of the IGFBP present in FFL. FFL from the second largest follicle of an individual cow immunized against GRF and treated with vehicle was subjected to SDS-PAGE under nonreducing conditions. The same sample was run in multiple lanes (2 μl per lane) on a single gel. After transfer to a PVDF filter, the lanes were cut apart and probed with [^{125}I]IGF-I (lane 2) or with antibodies against bovine IGFBP-3 (lane 3), bovine IGFBP-2 (lane 4), and human IGFBP-5 (lane 5) as described in Materials and Methods. Lane 1 represents serum (2 μl) obtained from this cow at 44 h after injection of vehicle, which was run on the same gel and probed with [^{125}I]IGF-I. Molecular weight standards were run in a parallel lane.

for all hormones analyzed. Therefore these treatments were combined for presentation (represented as VEH).

Mean concentrations of serum ST at 0 h were lower ($p < 0.01$) in GRFi (0.6 ± 0.2 ng/ml) than in HSAi (2.2 ± 0.2 ng/ml) cows (Fig. 1). However, 40 h after initiation of treatments, serum ST was similar ($p > 0.1$) in GRFi (10.0 ± 0.8 ng/ml) and HSAi (10.6 ± 0.8 ng/ml) cows (Fig. 1). Across immunization group, concentrations of ST were greater ($p < 0.05$) in serum from cows receiving rbST than in serum of cows receiving VEH (18.5 ± 1.2 vs. 2.0 ± 1.1 ng/ml, respectively; Fig. 1). The treatment-by-immunization interaction was not significant ($p > 0.1$). Both treatment by hour and immunization by hour affected ($p < 0.01$) serum ST concentrations.

Average serum concentrations of IGF-I at 0 and 40 h from initiation of treatments in GRFi and HSAi cows are depicted in Figure 2. Concentrations (ng/ml) of IGF-I were lower ($p < 0.01$) in GRFi (26.0 ± 4.0) than in HSAi (69.0 ± 5.0) cows at 0 h (Fig. 2). Serum IGF-I expressed as a percentage of respective 0-h concentrations was greater ($p < 0.05$) following rbST (269 ± 15) than following VEH (94 ± 15); this value was also greater in GRFi (362 ± 24) than in HSAi

(176 ± 16) cows receiving rbST (immunization by treatment, $p < 0.01$). Actual increases in serum IGF-I after rbST were 56 ± 11 ng/ml vs. 44 ± 11 ng/ml for GRFi and HSAi, respectively; and total concentrations at 40 h were not significantly different (54 ± 7 ng/ml vs. 88 ± 8 ng/ml, respectively, $p > 0.1$). The interactions of treatment by hour and immunization by hour contributed ($p < 0.01$) to variation in serum IGF-I.

Before treatment, serum concentrations of IGFBP-2 were significantly greater in GRFi than in HSAi cows (594 ± 48 vs. 384 ± 52 ng/ml, respectively; Fig. 3). After treatment with rbST, serum IGFBP-2 was decreased in both GRFi (371 ± 34 ng/ml) and HSAi (314 ± 27) cows (Fig. 3). Immunization by treatment was not significant ($p > 0.1$); however, both treatment by hour ($p < 0.05$) and immunization by hour ($p < 0.01$) contributed to variation in serum IGFBP-2.

Diameters of DOM or SUB were not affected ($p > 0.1$) by immunization or treatment (Table 1). As expected, mean diameter of DOM was greater ($p < 0.05$) than that of SUB.

Concentrations of IGF-I in FFL collected from GRFi or HSAi cows 44 ± 0.5 h after initiation of treatment are pre-

$M_r \times 10^{-3}$ **A**

$M_r \times 10^{-3}$ **B**
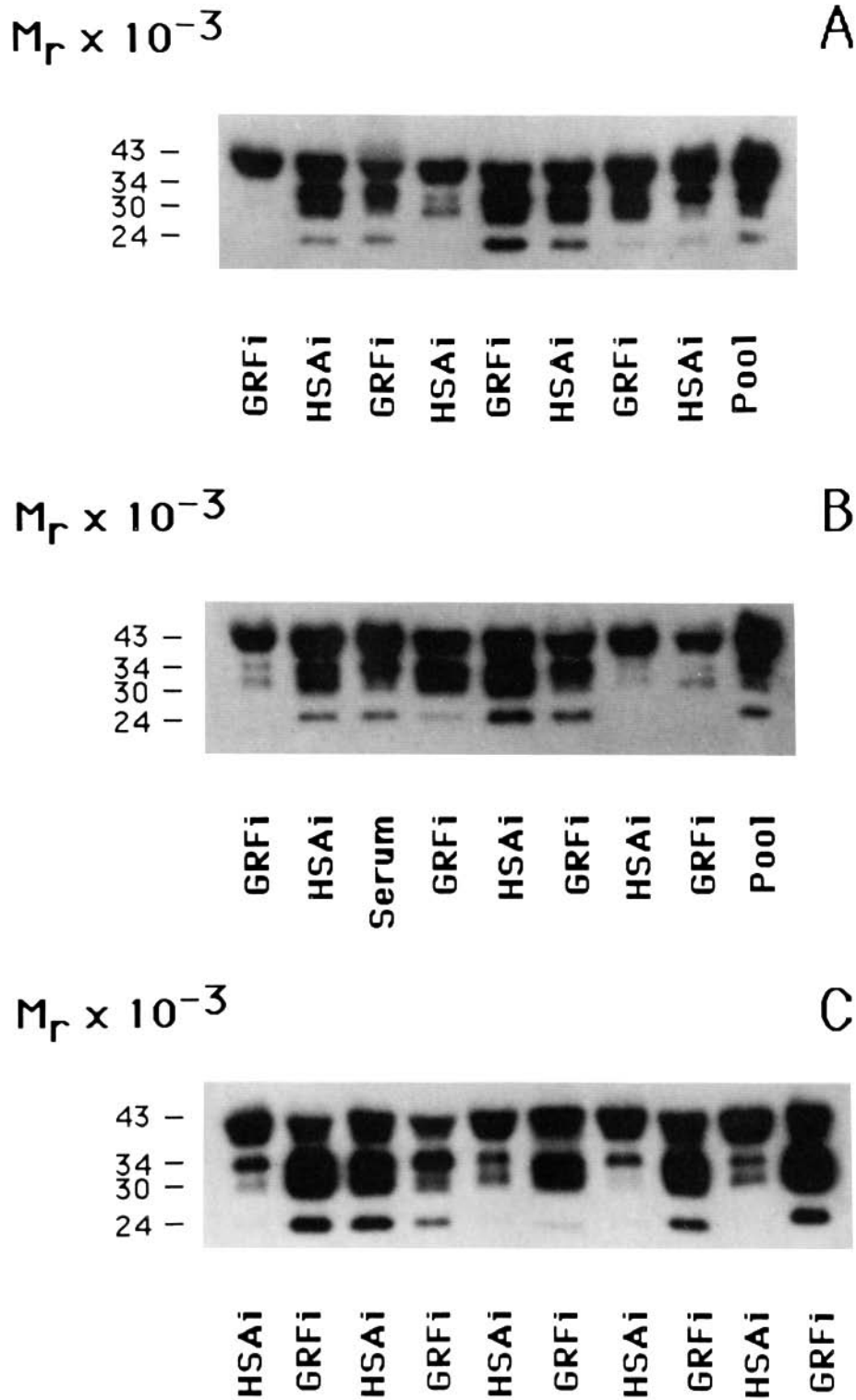
$M_r \times 10^{-3}$ **C**

FIG. 6. Forms of IGFBP present in FFL from individual largest (panel A and B) or second largest (panel C) follicles from cows immunized against GRF-(1–29)-Gly-Gly-Cys-NH$_2$-human serum albumin (GRFi) or human serum albumin (HSAi) and treated with vehicle only. Vehicle was administered (i.m.) at 0 and 24 h after PGF$_{2\alpha}$. All cows were unilaterally ovariectomized at 44 h. Serum from a single HSAi-vehicle-treated cow and from a bovine serum pool were included for comparison (panel A and B). Samples were subjected to SDS-PAGE under nonreducing conditions, transferred to PVDF filters, and probed with [^{125}I]-IGF-I as described in *Materials and Methods*. Molecular weight standards were run in a parallel lane.
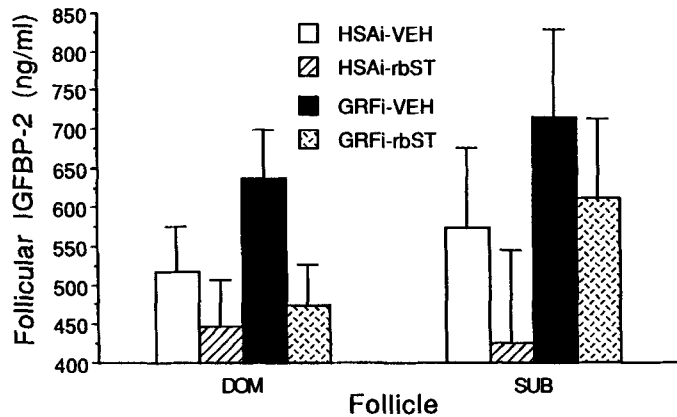
FIG. 7. FFL concentrations of IGFBP-2 (least-squares mean ± SE) in the largest (DOM) and second largest (SUB) follicles at 44 h after treatment with rbST, eCG, rbST and eCG, or vehicle in cyclic cows previously immunized against GRF-(1–29)-Gly-Gly-Cys-NH_2-human serum albumin (GRFi, n = 26) or human serum albumin (HSAi, n = 24). Treatments were administered (i.m.) at 0 and 24 h after prostaglandin $F_{2\alpha}$. All cows were unilaterally ovariectomized at 44 h. FFL concentrations of IGFBP-2 were similar in rbST and rbST-eCG-treated cows; thus data were combined and are presented as rbST (n = 25). Similarly, IGFBP-2 was similar in vehicle- and eCG-treated cows and data were combined for presentation (as VEH, n = 25). Follicular IGFBP-2 was greater (p < 0.05) in GRFi than in HSAi cows in SUB, but not in DOM. Concentrations of follicular IGFBP-2 from DOM and SUB were lower (p < 0.05) in rbST- than in VEH-treated cows. The treatment-by-immunization interaction was not significant.

sented in Figure 4. FFL concentrations of IGF-I (ng/ml) in DOM and SUB were lower ($p < 0.05$) in GRFi (88.6 ± 8 and 108 ± 11, respectively) than in HSAi (107 ± 6 and 127 ± 8, respectively) cows (Fig. 4). Regardless of immunization status, treatment (rbST vs. VEH) increased IGF-I (ng/ml) in DOM (110 ± 11 vs. 85 ± 9) and in SUB (134 ± 13 vs. 98 ± 12; Fig. 4, $p < 0.01$). The immunization-by-treatment interaction did not contribute ($p > 0.1$) to variation in FFL IGF-I. Follicular concentrations of IGF-I and follicular diameter were not correlated in either DOM ($r = -0.26$; $p = 0.07$) or SUB ($r = 0.08; p > 0.6$). However, serum IGF-I was correlated ($p < 0.01$) with FFL IGF-I in both DOM ($r = 0.64$) and SUB ($r = 0.44$). Across treatment and follicle, FFL IGF-I was correlated ($p < 0.05$) with follicular diameter ($r = -0.24$).

Ligand blot analysis of FFL and serum revealed multiple bands that bound [^{125}I]IGF-I (Fig. 6). Immunoblot analyses with specific antibodies against IGFBPs identified the 43 000 and 39 000 M_r bands as IGFBP-3; the 34 000 M_r band as IGFBP-2; and a 31 000–32 000 M_r doublet as IGFBP-5 (Fig. 5). The additional band at M_r 24 000 is likely to be IGFBP-4, but no immunoblot was conducted to verify this. Immunoblot analysis also indicated that FFL from both DOM and SUB contained two non-IGF binding IGFBP-2 fragments with approximate molecular weights of 22 000 and 14 000. Small amounts of IGFBP-5 fragment (approximately 21 000 M_r) were also detectable in some samples. The relative intensities of the IGFBP-2, -5, and -4 bands present in FFL from DOM were not different between GRFi and HSAi cows (Fig.

6, panels A and B), and considerable variation was observed between animals. However, with the exception of band intensities for one cow from each group, the intensities of the IGFBP-2, -5, and -4 bands were markedly greater in FFL from SUB of GRFi compared to HSAi animals (Fig. 6, panel C). In contrast, the band intensities of IGFBP-3 determined by scanning densitometry were lower for GRFi compared to HSAi animals with respect to FFL from both DOM (12 606 ± 2665 vs. 21 107 ± 2909; $p < 0.05$) and SUB (18 485 ± 316 vs. 28 658 ± 2793; $p < 0.05$).

Quantitation of IGFBP-2 by RIA indicated that active immunization against GRF increased ($p < 0.05$) IGFBP-2 (ng/ml) in FFL from SUB (664 ± 51 vs. 512 ± 51) but not ($p > 0.1$) from DOM (549 ± 29 vs. 482 ± 29; Fig. 7). Follicular IGFBP-2 (ng/ml) concentrations were decreased in both DOM (465 ± 38 vs. 560 ± 44) and SUB (536 ± 66 vs. 666 ± 66, $p < 0.05$) by rbST treatment. The immunization-by-treatment interaction was not significant ($p > 0.1$) for FFL IGFBP-2 in DOM or SUB. Follicular IGFBP-2 concentration and diameter were inversely related ($p < 0.05$) in both DOM ($r = -0.35$) and SUB ($r = -0.31$). Moreover, across treatment and follicle, FFL IGFBP-2 concentration and diameter were also negatively correlated ($p < 0.01; r = -0.30$). Serum concentration of IGFBP-2 was correlated ($p < 0.01$) with FFL IGFBP-2 only in DOM ($r = 0.57$). However, FFL IGF-I and FFL IGFBP-2 concentrations were not related ($p > 0.2$) in either DOM ($r = -0.18$) or SUB ($r = -0.17$). Source (DOM, SUB, or serum) contributed ($p < 0.01$) to variation in concentrations of IGFBP-2; however, source did not interact ($p > 0.2$) with treatment or immunization. Across treatment and immunization, concentrations of IGFBP-2 (ng/ml) were greater ($p < 0.05$) in SUB (599 ± 81) than in DOM (510 ± 58); this finding was similar to that for IGFBP-2 in serum (452 ± 38).

DISCUSSION

Active immunization against GRF provides an effective means to uncouple GRF and ST [4]. Immunoneutralization of GRF abolishes episodic release of ST and suppresses serum ST and IGF-I in cyclic gilts [38], lactating sows [39], lactating cows [27], growing steers [2, 40], and heifers [3]. Administration of booster immunizations to GRFi cows maintained suppressed serum ST and IGF-I throughout the experiment (Fig. 1 and 2).

In the present study, administration of rbST increased serum ST above physiological levels at 40 h after initiation of treatment in cows with normal (HSAi) or suppressed (GRFi) ST (Fig. 1). As would be expected, cows receiving rbST had increased concentrations of serum IGF-I by Hour 40; this increase elevated serum IGF-I to similar absolute levels in GRFi and HSAi cows (Fig. 2). The greater percentage increase in serum IGF-I observed in GRFi cows receiving rbST may indicate that GRFi cows are more sensitive to rbST. Alternatively, the observation that the absolute

increase in IGF-I was similar in GRFi and HSAi cows may indicate that capacity for IGF-I synthesis and secretion is not altered by GRFi. Future studies will be required to clarify these possibilities.

Delayed puberty has been observed in GRFi heifers in association with low serum IGF-I [3, 4] and in plerocercoid-treated female rats in association with depressed serum ST [41]. In the present study, active immunization against GRF lowered FFL concentrations of IGF-I in the DOM and SUB follicle; however, magnitude of difference was not as pronounced as that observed in serum (Figs. 2 and 4). Since IGF-I has been proposed as a local regulator of ovarian function [7, 9], a decrease in FFL IGF-I may also be involved in the delay of puberty observed in GRFi heifers. It is equally possible that GRFi may decrease ST in FFL to levels similar to that observed in serum. On the basis of previous reports [43], one would expect transudation of ST from serum to FFL. Although FFL ST concentration was not measured in the current study, our laboratory has demonstrated the presence of GRF antibodies in FFL, at levels > 90% of that detected in serum, aspirated from GRFi cows [42]. We therefore speculate that FFL ST would be correlated to serum ST concentration in the present study.

DOM taken from cows receiving rbST had greater concentrations of IGF-I regardless of immunization status (Fig. 4). This agrees with studies in gilts in which administration of porcine pituitary ST increased FFL and serum IGF-I concentrations [22, 24, 25]. Hammond et al. [44] reported that an increase in FFL IGF-I concentration is associated with increased follicular growth. Additionally, FFL IGF-I levels have been shown to increase with follicular diameter [44, 45], and twinning in cattle has been suggested to be associated with elevated serum and FFL IGF-I [46]. Neither a correlation between follicular diameter and IGF-I nor a treatment-by-diameter interaction was significant in the present study. These observations are in contrast to results in the literature [44–47], possibly because the majority of follicles examined in the current study would be classified as large ($\geq$ 8 mm) follicles. However, Howard and Ford [48] failed to demonstrate strong correlations between follicular diameter, estradiol, and follicular IGF-I in weaned sows. Co-treatment with ST and gonadotropins for human in vitro fertilization and embryo transfer has been shown to increase FFL IGF-I concentrations, number of oocytes collected, fertilization rate, and number of oocytes cleaved [49]. These data collectively support a role for FFL IGF-I in regulating folliculogenesis; however, conclusive evidence remains elusive.

It is important to determine the origin of FFL IGF-I. Follicular IGF-I could originate from serum (via transudation), and levels observed could be a reflection of systemic effects [43]. This is supported by the significant correlation between serum and FFL IGF-I concentrations observed in the present study. However, several studies have shown IGF-I to be produced within the ovary of rats [50–52], cows [53],

pig [9], and humans [54]. In addition to the evidence for ovarian synthesis of IGF-I, it has been shown that IGF-I secretion by porcine granulosa cells in vitro is enhanced by gonadotropins, estradiol, and ST [11, 55]. Greater differences in serum than in FFL after rbST treatment between GRFi and HSAi cows in the current study suggest differential regulation of serum and follicular levels of IGF-I (Figs. 2 and 4). Spicer et al. [56] observed that the ratio of FFL to plasma IGF-I tended to be greater in follicles from late (Days 3–6) than from early (Days 1 or 2) estrus in mares. Furthermore, short-term fasting (48 h) of Hereford × Fresian heifers [57] or feed restriction of cycling cows [42] has been reported to significantly decrease plasma IGF-I concentrations without affecting IGF-I concentrations in large follicles. These data suggest that under certain physiologic conditions, IGF-I concentrations may be regulated locally.

In the current study, eCG did not affect follicular IGF-I or IGFBP-2 concentrations. The lack of an effect may indicate an insufficient eCG dosage resulting from our desire to avoid superovulation. However, the eCG used in this study was shown to be biologically active via increased ovulation rate in mice [29]. Concentrations of estradiol and P_4 in FFL could not be determined because of inadequate sample volume. It has been indicated that the ovarian IGF system functions as an important local amplification mechanism for steroidogenesis and gonadotropin action [7, 58]. Hsu and Hammond [11] have reported enhanced secretion of immunoreactive IGF-I by cultured porcine granulosa cells when treated with LH and/or FSH. In two separate studies, follicular IGF-I concentrations have been reported to increase during eCG-induced follicular growth in gilts [24, 59]. Adashi et al. [60] have shown the ability of FSH to regulate granulosa cell IGFBP in a dose- and time-dependent, but biphasic, manner. Studies conducted in rodents concerning the effects of eCG on follicular IGF-I are difficult to interpret, as such effects may be species-specific. Spicer et al. [45] reported minimal changes in IGF-I levels during spontaneous or GnRH-induced follicular development in postpartum cattle.

Since IGFBP modulate IGF function, defining how they are regulated in the ovary is critical to understanding changes in ST-IGF-I physiology. Through immunoblotting of FFL, three forms of IGFBP were identified as IGFBP-3, -2, and -5. It is most likely that the band migrating at 24 000 M_r is IGFBP-4, as this IGFBP has also been identified in human FFL [61]. All three forms were present in FFL from both DOM and SUB follicles. Multiple IGFBP species have been reported previously in FFL from murine and human FFL [62–64], and IGFBP-2 through -6 have been detected in porcine FFL [65]. Immunoblotting analysis also indicated the presence of IGFBP-2 and -5 fragments corresponding in size with fragments that have been identified in serum of neonatal fasted pigs [66] as well as in media conditioned by pig smooth muscle cells [67] and by human fibroblasts [36]. Fragment formation of IGFBP-5 in conditioned media of

human fibroblasts has been shown to be regulated by IGF-I. Whether such a mechanism is operative for any form of IGFBP at the level of the ovary remains to be determined.

In contrast to serum ST and IGF-I concentrations, circulating IGFBP-2 concentrations were elevated in GRF-immunized cows. This is consistent with observations that serum IGFBP-2 is elevated in humans [68] and in transgenic mice [69] with ST deficiency. In the present study, rbST decreased serum IGFBP-2 in cows with normal (HSAi) and suppressed (GRFi) serum ST while simultaneously increasing serum IGF-I (Fig. 3). These data suggest that serum ST and/or IGF-I are mediators of serum IGFBP-2 concentrations. Treatment with rbST has also been reported to significantly decrease serum IGFBP-2 in lactating dairy cows [34, 70]. In contrast, infusion of IGF-I has been shown to induce IGFBP-2 in humans in vivo [71, 72]. The mechanism(s) by which either ST or IGF-I mediates serum IGFBP-2 concentrations is presently unknown.

Concentrations of IGFBP-2 were elevated in both serum and FFL from SUB follicles of cows immunized against GRF, and treatment with rbST decreased IGFBP-2 in serum as well as in FFL from DOM and SUB follicles of both GRFi and HSAi cows. Also, average concentrations of IGFBP-2 were greater in FFL from SUB than in FFL from DOM follicles or in serum. Collectively these data, coupled with the observation that IGFBP-2 is negatively correlated with follicular diameter, indicate that IGFBP-2 is synthesized at a higher rate in small follicles. However, all compartments (DOM, SUB and serum) may have been affected by changes in serum IGFBP-2, because IGFBP-2 decreased in all compartments following rbST. In contrast, GRFi increased levels of IGFBP-2, -5, and -4 in FFL of SUB but not DOM, suggesting that regulation of these IGFBP in DOM may be occurring locally at the tissue level. Ovarian tissue mRNA for IGFBP-2 through -5 has been identified in the rat [73, 74], for IGFBP-2 and -3 in the pig [75], and for IGFBP-1 through -3 in the human [76]. Furthermore, cultured granulosa and thecal cells have been shown to synthesize IGFBP [76–78, 62]. Therefore, changes in local production of IGFBP have the potential to play a key role in regulating the availability of IGF to ovarian cells. Addition of exogenous IGFBP has been shown to inhibit the ability of FSH and IGF-I to stimulate steroidogenesis, cAMP generation, and DNA synthesis in cultured granulosa cells [12, 79, 80]. Recently, IGF analogues with reduced affinities for IGFBP but not the Type-I receptor were shown to be more effective than IGF-I in enhancing FSH-stimulated accumulation of P_4 by rat granulosa cells [81] and in stimulating IGFBP synthesis [82]. Since FSH also has been shown to inhibit the secretion of IGFBP by granulosa cells [61, 62, 77], this may represent a mechanism by which IGF availability is increased to promote granulosa cell growth. Furthermore, activation of Type-I IGF receptors in porcine granulosa cells by IGF-I results in increased synthesis and release of IGFBP, which, in turn, appear to sequester and neutralize IGF-I [82]. However, several forms of IGFBP have

also been shown to stimulate IGF bioactivity in other cell types under certain conditions [83]. Therefore, the exact role of the IGFBP in modulating IGF activity at the level of the ovary in vivo remains unknown.

GRFi increased the relative amounts of IGFBP-2, -4, and -5, while decreasing the amount of IGFBP-3 in FFL from DOM and SUB. Nakatini et al. [73] and Erickson et al. [74] have reported IGFBP-4 and -5 mRNA to be localized almost exclusively to granulosa cells of atretic follicles. IGFBP-2, -4, and a 29 000 M_r IGFBP were also significantly increased in FFL from women with polycystic ovarian disease [61], while no difference in IGFBP-3 levels was reported. Therefore, the various forms of IGFBP could play different roles in regulating follicular growth.

It is apparent that ST, IGF-I, and IGFBP are interrelated in serum and FFL of cattle. The present study has provided evidence suggesting that IGF-I and IGFBP are differentially regulated in DOM and SUB follicles in cyclic beef cows. How these hormones participate in attainment of puberty, folliculogenesis, and ovarian function is not understood, particularly in vivo. Future studies are needed to clarify how interactions between IGF-I and IGFBP affect follicular growth.

ACKNOWLEDGMENTS

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