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Relationships among Concentrations of Steroids, Insulin-like Growth Factor-I, and Insulin-like Growth Factor Binding Proteins in Ovarian Follicular Fluid of Beef Cattle[1]

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ABSTRACT

The relationship between ovarian follicular steroidogenesis and insulin-like growth factor binding protein (IGFBP) activity was evaluated during the follicular phase of the bovine estrous cycle. In experiment 1, follicles were collected from cyclic cows (n = 11) slaughtered at 48 h after administration of prostaglandin $F_{2\alpha}$ (PGF; 35 mg i.m.). In experiment 2, cows were injected twice daily with saline (control) or FSH (FSH cows; total dosage = 42 mg) from Day 2 to Day 6 (estrus = Day 0) and with PGF (35 mg i.m.) on Day 7; follicles were collected from control cows (n = 20) slaughtered at 0, 24, 48, or 72 h and from FSH cows (n = 8) at 0 and 48 h after PGF. Follicular fluid was assayed for estradiol (E_2), androstenedione (A_4), progesterone (P_4), and insulin-like growth factor-I (IGF-I) by RIA and for IGFBP activity by ligand blotting and densitometry. Intensities of the 34-kDa (IGFBP-2), 29–27-kDa, and 22-kDa IGFBP bands in follicular fluid were nondetectable or were lower ($p < 0.01$) in the fluid of large (≥ 8 mm) E-active (E-A; $E_2 > 50$ ng/ml and $> P_4$) follicles than in large E-inactive (E-I), medium (5–7 mm), or small (< 5 mm) follicles. IGFBP-3 (44–40-kDa doublet) was unaffected by follicle stage in experiment 1, but IGFBP-3 was lower ($p < 0.01$) in follicular fluid of E-A vs. E-I large follicles in experiment 2. Profiles of IGFBP activity were similar in follicular fluid of small, medium, and E-I large follicles. In experiment 2, E_2 concentrations in large E-A follicles increased ($p < 0.01$) from 0 to 48 h after the PGF injection for control cows but decreased ($p < 0.01$) for FSH cows, whereas follicular fluid IGFBP-2 binding activity decreased from 0 to 48 h after PGF in controls and increased in FSH cows (treatment × time, $p < 0.05$). IGFBP-3 binding was unaffected by FSH treatment or time after administration of PGF. Profiles of IGFBP activity in homogenates of granulosa or theca cells were similar to follicular fluid profiles except for the absence of IGFBP-3 binding activity. The disappearance of binding activities for IGFBP-2 and smaller-molecular-mass IGFBPs in E-A follicles suggests a possible regulatory role for IGFBPs in follicular maturation and on aromatase activity. Likewise, the elevated E_2 secretion in large follicles recruited by FSH in vivo coincided with a reduction in binding activity of IGFBPs (i.e., ≤ 34 kDa) in the follicular fluid.

INTRODUCTION

In vitro studies with mammalian ovarian tissue have provided evidence that insulin-like growth factor-I (IGF-I) stimulates the proliferation of follicular cells and enhances gonadotropin-stimulated steroidogenesis in both follicular and luteal cells [1, 2]. Although the liver is the proposed major source for IGF-I measured in blood, mRNA for IGF-I has been identified in ovarian tissue from rats [3, 4], cattle [5, 6], swine [7], and sheep [8]. However, the relative contribution of absorption vs. intrafollicular secretion of IGF-I to concentrations of IGF-I in bovine follicular fluid is unknown [9].

Assessment of the biological actions of IGFs on mammalian cells in vivo is complicated by the presence of specific, high-affinity binding proteins for IGFs, ranging in molecular mass from 21 to 150 kDa, in biological fluids. To date, six insulin-like growth factor binding proteins (IGFBP) have been identified in human ovaries [10, 11] and five in rat ovaries [12, 13] by Western immunoblot analysis, Northern blot analysis, and in situ hybridization procedures. Nomenclature and approximate molecular masses of the human IGFBPs are IGFBP-1, 28 kDa; IGFBP-2, 34 kDa; IGFBP-3, 40 and 43 kDa; IGFBP-4, 24 and 30 kDa; IGFBP-5, 29–31 kDa; and IGFBP-6, 22 kDa [14]. Depending on the circumstances, these IGFBP can either inhibit [15] or stimulate [16] the action of IGF-I on mammalian cells, but their predominant effect on ovarian follicular cells in vitro has appeared to be inhibition [2, 17]. Interestingly, mRNA for two of the IGFBP (i.e., IGFBP-4 and -5) in the rat [18, 19] and human [11] ovary have been localized in the granulosa of atretic follicles.

As follicular cells are a proposed site of production and action for IGFs and IGFBPs, we hypothesize that intrafollicular IGFBP production and/or content modulate the action of IGFs on ovarian folliculogenesis in cattle via autocrine/paracrine regulation. Thus, the objective of the present research was to relate qualitative and quantitative changes in IGFBP binding activity to the physiological status of bovine ovarian follicles.

MATERIALS AND METHODS

Animals and Experimental Procedures

Experiment 1. Cyclic beef cows (n = 11) were administered 35 mg of prostaglandin $F_{2\alpha}$ (PGF, Lutalyse; The Upjohn Company, Kalamazoo, MI) i.m. during the luteal

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[1]Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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phase of the estrous cycle to induce luteolysis. The animals were slaughtered 46–48 h after the PGF injection to ensure the presence of the preovulatory follicle. Both ovaries were removed immediately and stored on ice. Follicles > 5 mm in diameter were dissected from the surface of the ovaries; the follicular fluid was aspirated with a syringe and hypodermic needle and stored separately for each individual follicle at −70°C until assayed. A sample of jugular vein blood was collected at slaughter and stored on ice until centrifuged; serum was decanted and stored at −20°C.

Experiment 2. Cyclic beef cows were injected twice daily with saline (control cows, n = 20) or with 6, 5, 4, 3, and 3 mg of FSH per injection on Day 2, 3, 4, 5, and 6 of the estrous cycle (estrus = Day 0), respectively, for a total dosage of 42 mg (FSH cows, n = 8). An injection of PGF was administered on Day 7 after estrus, 24 h after the last FSH treatment. Control cows were slaughtered at 0, 24, 48, or 72 h after the PGF injection, and FSH cows were slaughtered at 0 or 48 h after PGF to enable comparison of follicular fluid components among follicles at the luteal, proestrous, and estrous phases of the estrous cycle. Ovaries were collected immediately; all visible follicles were dissected from the ovaries of controls, whereas four to five large (≥ 8 mm in diameter) follicles were selected randomly from the ovaries of FSH-treated cows. Follicular fluid was aspirated with a syringe and hypodermic needle and stored at −70°C until assayed. Follicular fluid from large follicles was collected and stored individually, but samples from medium (5–7 mm) and those from small (< 5 mm) follicles were pooled for each cow. A sample of jugular vein blood was collected immediately before slaughter.

Analysis of Hormones and IGF Binding Proteins

Concentrations of estradiol (E_2), androstenedione (A_4), and progesterone (P_4) in ovarian follicular fluid were measured by RIA procedures as described previously [9] except that ^{125}I-radiolabeled ligands were used and the ligand-antibody complex was precipitated with preprecipitated anti-rabbit antibody. The intraassay and interassay coefficients of variation for two E_2, A_4, and P_4 RIAs were 7.2 and 11.4%, 8.1 and 14.5%, and 9.2 and 13.2%, respectively. Follicular fluid samples were assayed directly for P_4, whereas serum samples were extracted initially with heptane. The extraction and RIA procedures for quantitating IGF-I (i.e., total IGF-I) in follicular fluid and serum have been described previously [9]. The intraassay coefficient of variation was 6.7%.

A qualitative and quantitative evaluation of IGFBPs in bovine serum, follicular fluid, and follicular cells was conducted by ligand blot analysis as described by Howard and Ford [20] with a few modifications. Proteins were separated by one-dimensional SDS-PAGE using 4% stacking gel and 14% polyacrylamide separating gel. One microliter of follicular fluid or serum plus 24 μl of sample buffer was loaded per lane. A serum standard pool was included in lane 1 of each gel to enable comparisons among gels within an experiment. Proteins were electrophoretically transferred from gels to nitrocellulose membranes. Membranes were probed overnight with ^{125}I-IGF-I and subsequently exposed to x-ray film for 48 or 96 h at −70°C to detect IGFBP activity. Band intensity on autoradiographs was quantitated by scanning densitometry. Serial dilutions of serum or follicular fluid in loading buffer resulted in linear quantitative reductions in band intensities.

Ligand blot analysis was used to determine the presence of IGFBP binding activity in follicular cell extracts of individual large follicles and pools from medium and small follicles from one cow. Granulosa cells were scraped from the wall of the antrum with a nylon loop, and theca cells were then dissected microscopically from the basement membrane. The isolated cells were washed in DMEM/F-12 and homogenized in 400 μl of 0.1% SDS, 1% cholate, and 0.1 mM PMSF buffer; 200 μg protein (BCA procedure; Pierce, Rockford, IL) was loaded per lane.

Bands for IGFBP-2 and -3 were identified by immunoblot analysis [21] on duplicate blots of selected samples. Antiserum to rat IGFBP-2 was obtained from Dr. George Veomett, University of Nebraska, Lincoln, NE. As shown in Figure 2, the antiserum to bovine IGFBP-3 (RK53), obtained from Dr. David Clemmons, University of North Carolina, Chapel Hill, NC, cross-reacted with IGFBP-2 also.

Statistical Analysis

Data were analyzed by least squares analysis of variance by the GLM procedure of SAS [22]. Large follicles were further classified as either E-active (E-A; E_2 > 50 ng/ml and E_2:P_4 ratio > 1) or E-inactive (E-I). Because of heterogeneous variances among follicular stages, analyses of E_2, IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP data were performed on transformed ($\log_{10} \times + 1$) data. The statistical model for experiment 1 evaluated the effect of follicle stage (medium and E-A or E-I large follicles) on the nine follicular variables measured (Table 1); cow × stage was the error term. Residual and simple (Pearson) correlation coefficients were calculated by multivariant analysis of variance and linear regression analysis (weighted and unweighted for follicular diameter), respectively, to assess relationships among the nine follicular variables. Reported simple correlation coefficients were weighted for follicular diameter except for relationships with follicle diameter.

The effect of time after PGF injection on follicular fluid variables in experiment 2 was evaluated in two models. The first model evaluated the effects of time after PGF (0, 24, 48, or 72 h), follicle stage (small, medium, and E-A or E-I large follicles), and time × stage on follicles from controls only; error terms were cow within time for time after PGF, and cow within time × follicle stage to test stage and stage × time. For comparisons between control and FSH-treated cows the model was treatment, time after PGF (0 vs. 48), follicle stage (E-A vs. E-I), and all two- and three-way interactions. Cow within treatment × time was the error term

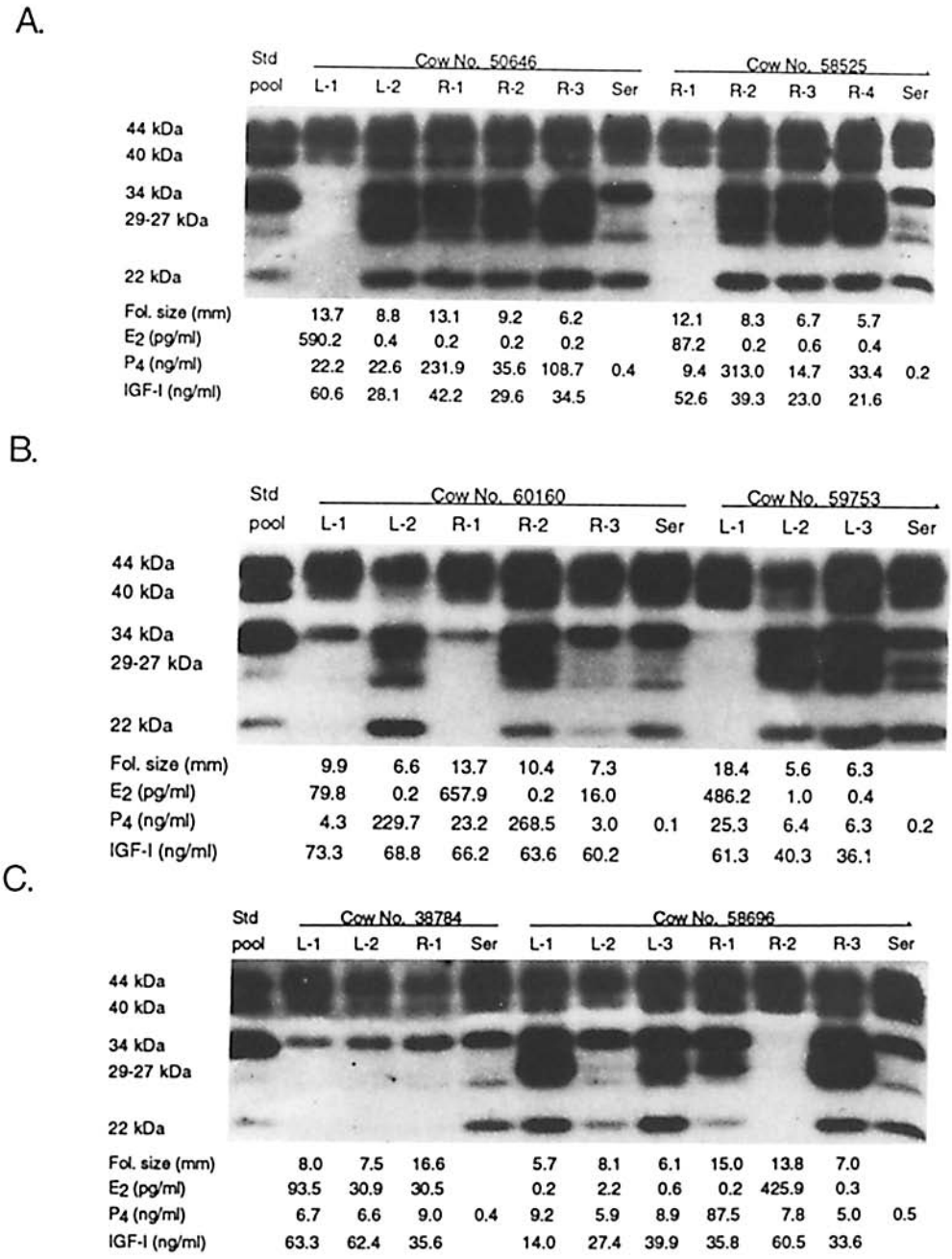

FIG. 1. Ligand blots of IGFBP binding activity in follicular fluid of individual ovarian follicles collected from cows slaughtered 48 h after PGF (experiment 1). Diameter (fol. size) and concentrations of E_2, P_4, and IGF-I are listed below each follicle. A standard serum sample (Std pool), in which the IGFBP-3 (44–40 kDa) and IGFBP-2 (34 kDa) bands were identified by immunoblot analysis, was included in lane 1 of each blot. Follicular fluid and serum (ser) samples were grouped by animal in adjoining lanes. Follicular fluid identification (e.g., L-1) includes location of ovary (left vs. right) and follicle ID.

to test the effects of treatment, time, and treatment × time; and follicle stage × cow within treatment × time was the error term to test stage and its interactions. Residual and simple correlations were calculated as described for experiment 1. Reported simple correlations were weighted for follicle diameter with the exception of correlations with follicle diameter. The only significant ($p < 0.05$) residual correlations were among IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP.

RESULTS

Experiment 1

Relationships among IGFBP profiles (ligand blots), steroid concentrations, and IGF-I concentrations in follicular

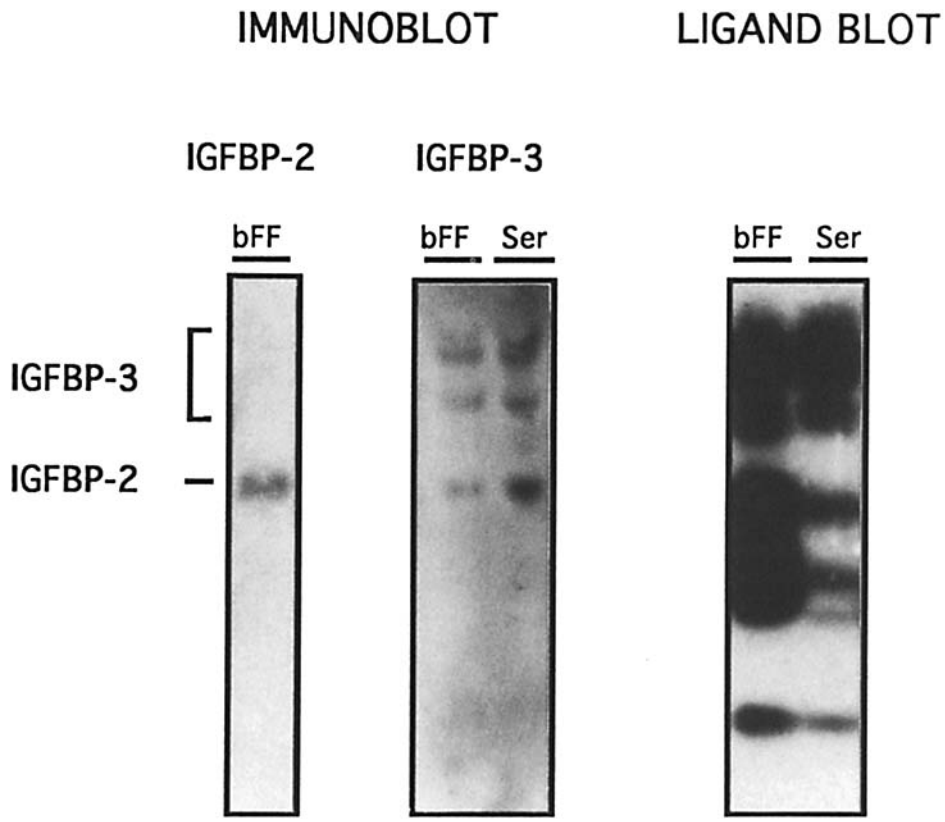

FIG. 2. Immunoblots and ligand blot of duplicate samples of bovine follicular fluid (bFF) and/or serum (ser). The specific IGFBP-2 antiserum was produced to rat IGFBP-2; the IGFBP-3 was produced to bovine IGFBP-3, which was contaminated with IGFBP-2, resulting in cross-reaction with IGFBP-2.

fluid of individual follicles and in serum at 48 h after PGF are illustrated in Figure 1 for six of the cows. The ligand blots contained major bands of IGF-I binding at 44, 40, 34, 29–27, and 22 kDa. Western immunoblot analysis identified the 34-kDa band as IGFBP-2 and the 44- and 40-kDa bands as doublets of IGFBP-3 (Fig. 2). Means (± SEM) for concentrations of steroids, IGF-I, and IGFBP binding activity are reported in Table 1 for large E-A, large E-I, and medium follicles; number of follicles per stage obtained from each cow ranged from 1 to 2, 0 to 2, and 1 to 6, respectively. Large preovulatory (i.e., E-A) follicles had higher ($p < 0.01$) concentrations of E_2 and IGF-I but lower ($p < 0.01$) IGF-I

TABLE 1. Effect of follicular stage on concentrations of steroids, IGF-I, and IGFBP in follicular fluid 48 h after PGF treatment (experiment 1).[a]

Variables	Large E-A[b]	Large E-I[b]	Medium (5–7 mm)
No. follicles[c]	13 (1–2)	12 (0–2)	28 (1–6)
Follicle diameter (mm)	13.5 ± 0.8[e]	11.9 ± 0.8[e]	6.5 ± 0.7[f]
Estradiol (ng/ml)	344.8 ± 36.2[e]	7.0 ± 37.6[f]	7.9 ± 30.7[f]
Androstenedione (ng/ml)	72.2 ± 16.7	28.3 ± 17.4	46.6 ± 14.2
Progesterone (ng/ml)	15.8 ± 10.3	39.5 ± 10.8	10.3 ± 8.8
IGF-I (ng/ml)	61.4 ± 3.6[e]	40.6 ± 3.7[f]	45.9 ± 3.0[f]
IGFBP-3 (U/μl)	3.00 ± 0.47	3.81 ± 0.49	2.91 ± 0.40
IGFBP-2 (U/μl)	0.10 ± 0.43[e]	2.22 ± 0.45[f]	2.00 ± 0.37[f]
29–27 kDa[d] (U/μl)	0.01 ± 0.35[g]	1.18 ± 0.37[h]	0.95 ± 0.30[h]
22 kDa (U/μl)	0.00 ± 0.09[e]	0.45 ± 0.10[f]	0.34 ± 0.08[f]

[a]Mean ± SEM, n = 11 females.
[b]Large follicles (≥8 mm) were classified as estrogen-active (E-A) or -inactive (E-I); E-A = E_2 concentration > 50 ng/ml and E_2:P_4 > 1.
[c]Number of follicles per female is in parentheses.
[d]Sum of IGFBP bands in the 29–27-kDa region.
[ef]Means without a common superscript differ by follicular stage ($p < 0.01$).
[gh]Means without a common superscript differ by follicular stage ($p < 0.05$).

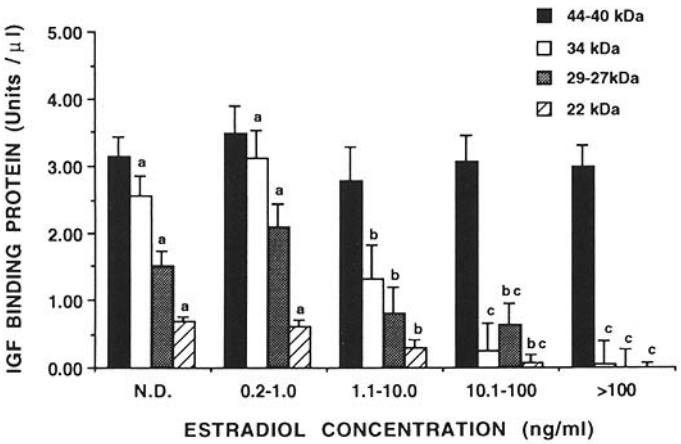

FIG. 3. Relationship between concentrations of E_2 and IGFBP binding activity in follicular fluid of individual ovarian follicles > 5 mm in diameter at 48 h after PGF. [abc]Means without a common superscript differ among E_2 groups ($p < 0.05$).

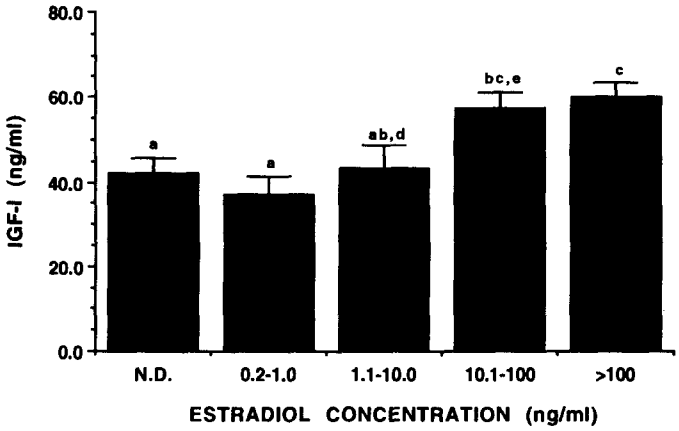

FIG. 4. Relationship between concentrations of E_2 and total IGF-I in follicular fluid of follicles (> 5 mm) collected 48 h after PGF (experiment 1). [abc]$p < 0.01$, [de]$p < 0.05$.

binding activity in the 34 (IGFBP-2)-, 29–27-, and 22-kDa IGFBP bands than large E-I or medium follicles. Concentrations of E_2 and IGFBP activity did not differ ($p > 0.10$) between large E-I and medium follicles. Concentrations of A_4, P_4, and IGFBP-3 binding did not differ ($p > 0.10$) among follicle stages.

A summary of IGFBP binding activity in follicular fluid relative to E_2 concentration is presented in Figure 3. For clarity, identification of statistical differences was limited to $p < 0.05$ in Figure 3. Follicles (> 5 mm) were categorized into five E_2 groups: nondetectable (< 0.2 ng/ml E_2), 0.2–1.0, 1.1–10.0, 10.1–100.0, and > 100 ng/ml. Intensity of the 34 (IGFBP-2)-, 29–27-, and 22-kDa IGFBP bands in follicular fluid decreased ($p < 0.01$) as E_2 concentration increased ($r = -0.49$, -0.34, and -0.43, respectively, for simple correlations weighted for follicle size; $p < 0.01$), whereas intensity of the 44–40-kDa (IGFBP-3) bands was unaffected by E_2 concentration ($r = -0.01$; $p > 0.10$). Specifically, binding activities for IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP were lower ($p < 0.01$) in follicular fluid of follicles with E_2 concentrations > 10.0 ng/ml than in follicles with E_2 concentrations ≤ 1.0 ng/ml. Binding activities (i.e., ≤ 34 kDa) in follicular fluid of follicles with E_2 concentrations of 1.1 to 10.0 ng/ml were intermediate ($p < 0.05$) between the values for follicles with nondetectable E_2 and values for those with > 100 ng/ml E_2 concentrations. In addition, the number of follicles containing detectable bands for these IGFBP decreased as E_2 concentrations increased. Total IGFBP binding activities (i.e., sum of all bands) also decreased ($p < 0.01$) as follicular fluid E_2 concentration increased; means for total IGFBP activity were 7.83, 9.80, 5.53, 4.24, and 3.06 units/ml for E_2 concentrations of < 0.2, 0.2–1.0, 1.1–10.0, 10.1–100, and > 100 ng/ml, respectively. The concurrent decline in binding activity for IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP resulted in positive coefficients for the simple correlations (weighted for follicle diameter) among the three binding activities; $r = 0.61$ and 0.77 for IGFBP-2 vs. 29–27- or 22-kDa IGFBP and $r = 0.72$ for 29–27- vs. 22-kDa IGFBP. Residual correlation coefficients were $r = 0.45$, 0.63, or 0.63 for IGFBP-3 vs. IGFBP-2, 29–27-kDa IGFBP, or 22-kDa IGFBP, respectively; $r = 0.52$ or 0.77 for IGFBP-2 vs. 29–27-kDa or 22-kDa IGFBP; and $r = 0.75$ for 29–27-kDa vs. 22-kDa IGFBP.

Conversely, concentrations of IGF-I in follicular fluid (Fig. 4) increased ($p < 0.01$) with increasing E_2 concentrations ($r = 0.48$; $p < 0.01$); IGF-I was greater ($p < 0.01$) in follicles with E_2 > 10.0 ng/ml than in follicles with E_2 ≤ 1.0 ng/ml (10.1–100 vs. 1.1–10.0 ng/ml; $p < 0.05$). Consequently, follicular fluid IGF-I concentrations were correlated negatively ($p < 0.01$) with intensity of the IGFBP-2 ($r = -0.51$), 29–27-kDa IGFBP ($r = -0.54$), and 22-kDa IGFBP ($r = -0.39$) bands.

Correlations among follicular fluid steroid concentrations were significant ($p < 0.01$) between E_2 and A_4 ($r = 0.47$), but not between E_2 and P_4 ($r = -0.11$) or between A_4 and P_4 ($r = -0.01$). Follicle size (diameter) influenced the concentration of E_2 ($r = 0.62$, $p < 0.01$), but not ($p > 0.10$) concentrations of A_4 ($r = 0.05$) or P_4 ($r = 0.05$) in follicular fluid.

Experiment 2

Control females. Representative ligand blots of IGFBP binding activity in follicular fluid of control and FSH cows are shown in Figure 5. Treatments and times were randomly distributed among gels. Each control cow had one large E-A follicle and one pool each of medium and small follicles; eight cows had one large E-I follicle (n = 3 at 0, 1 at 24, 2 at 48, and 2 at 72 h). As in experiment 1, intensities of the 34 (IGFBP-2)-, 29–27- and 22-kDa IGFBP bands were either nondetectable or were reduced significantly ($p < 0.01$) in the follicular fluid of large E-A follicles compared to large E-I, medium, or small follicles (Fig. 5 and Tables 2 and 4). Follicular fluid pools from small follicles

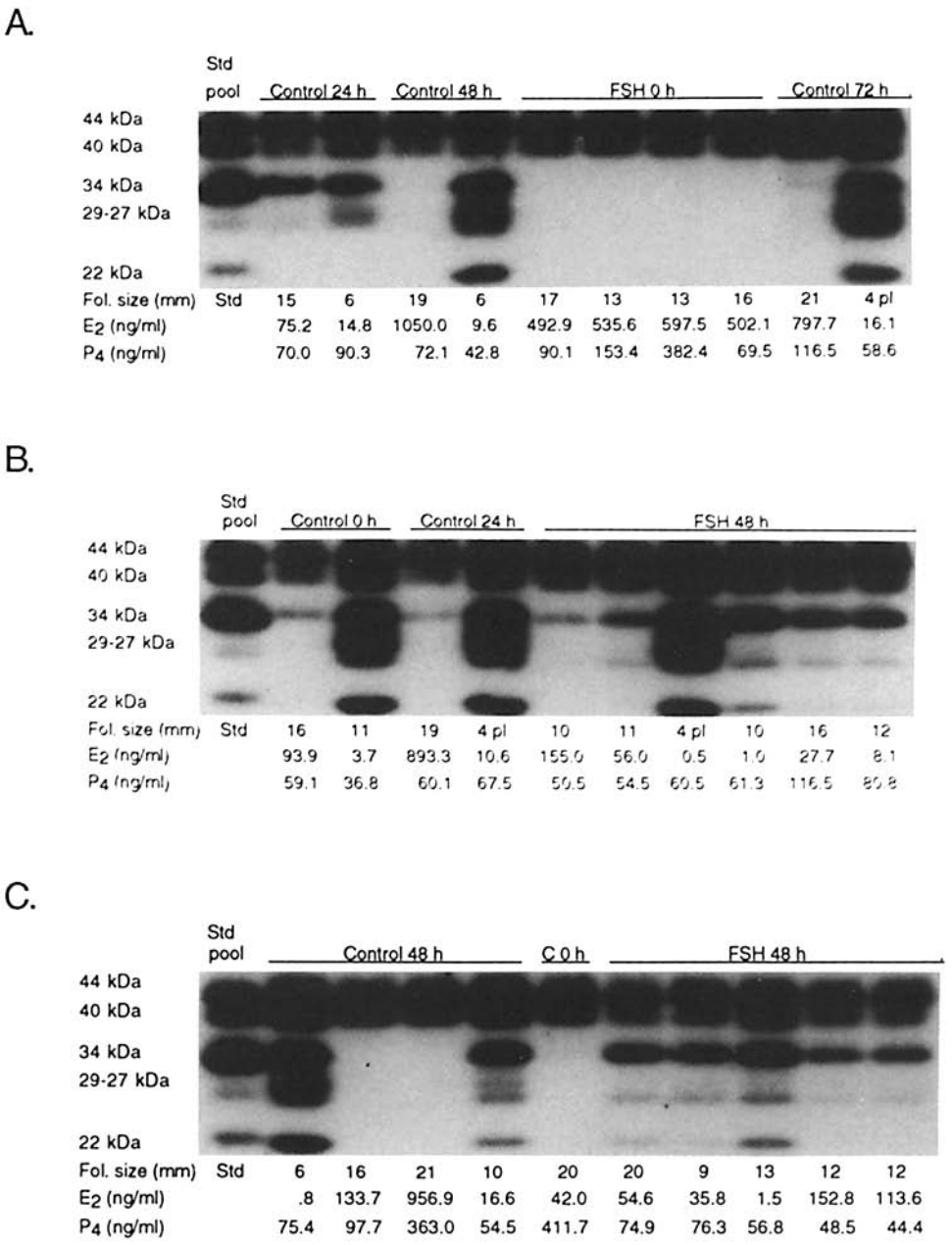

FIG. 5. Ligand blots of IGFBP binding activity in follicular fluid of individual ovarian follicles from cows treated with saline (control) or FSH (total dosage = 42 mg) for 5 days (experiment 2). Animals were slaughtered at 0, 24, 48, or 72 h after administration of PGF on the last day of treatment. Follicular fluid samples were grouped by animal, whereas treatments and times were distributed randomly among gels.

(Table 2) contained more ($p < 0.05$) IGFBP-2 and 22-kDa IGFBP than large E-I follicles, and more ($p < 0.01$) 29–27-kDa IGFBP than large E-I or medium follicles. Binding activity of IGFBP-3 was lower ($p < 0.01$) in follicular fluid of large E-A than in that of large E-I, medium, or small follicles. Simple correlation coefficients (weighted for follicle diameter) for the negative relationships between follicular fluid E_2 concentrations and binding activity of IGFBP-3, IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP for control cows were $r = -0.18$ ($p > 0.10$), -0.57 ($p < 0.01$), -0.43 ($p < 0.01$), and -0.47 ($p < 0.01$), respectively. Conversely, relationships among the binding proteins were positive ($p < 0.01$): $r = 0.53$, 0.43, and 0.52 for IGFBP-3 vs. IGFBP-2, 29–27-kDa IGFBP, or 22-kDa IGFBP; $r = 0.77$ and 0.85 for IGFBP-2 vs. 29–27- or 22-kDa IGFBP; and $r = 0.87$ for 29–27- vs. 22-kDa IGFBP, respectively. The respective residual correlations among IGFBP-2, 29–27-kDa, or 22-kDa IGFBP were 0.91, 0.81, and 0.77 ($p < 0.01$).

Changes in follicular fluid E_2 concentrations and IGFBP binding activity with time after PGF administration are re-

TABLE 2. Effect of follicle stage on concentrations of steroids, IGF-I, and IGFBPs in follicular fluid of control cows (experiment 2).[a]

Variables	Follicular stage[b]			
	Large E-A	Large E-I	Medium pools	Small pools
No. follicles[c]	20	8	20	20
Follicle diameter (mm)	17.5 ± 0.4[e]	13.0 ± 0.6[f]	5–7	≤4
Estradiol (ng/ml)	508.0 ± 42.6[e]	5.6 ± 67.4[f]	4.6 ± 42.6[f]	13.4 ± 42.6[f]
Androstenedione (ng/ml)	68.7 ± 9.6	33.8 ± 15.2[h]	89.2 ± 9.6[i]	62.2 ± 9.6
IGF-I (ng/ml)	66.0 ± 2.4[e]	46.6 ± 5.1[f]	57.9 ± 2.4[g]	53.9 ± 2.4[fg]
IGFBP-3 (U/μl)	2.25 ± 0.20[e]	3.61 ± 0.31[f]	3.15 ± 0.20[f]	3.80 ± 0.20[f]
IGFBP-2 (U/μl)	0.08 ± 0.13[e]	1.69 ± 0.20[fh]	2.21 ± 0.13[f]	2.46 ± 0.13[fi]
29–27 kDa (U/μl)[d]	0.01 ± 0.23[e]	1.35 ± 0.37[f]	1.71 ± 0.23[f]	4.02 ± 0.23[g]
22 kDa (U/μl)	0.01 ± 0.03[eh]	0.24 ± 0.05[ei]	0.50 ± 0.03[f]	0.93 ± 0.03[g]

[a]Mean ± SEM. Cows were slaughtered at 0, 24, 48, or 72 h after PGF (n = 5 cows per time) and statistical model included time as a covariate.
[b]Follicular fluid was collected from individual large (diameter ≥8 mm) follicles whereas follicular fluid of medium or small follicles were pooled within females; E-A = E_2 concentration > 50 ng/ml and E_2:P_4 > 1.
[c]Each cow was represented once in the large E-A, medium and small follicular stage; eight cows had one large E-I follicle.
[d]Sum of 29–27-kDa bands.
[efg]Means without a common superscript differ by follicular stage ($p < 0.01$).
[hi]Means without a common superscript differ by follicular stage ($p < 0.05$).

ported in Table 3 for control cows. E_2 concentrations in follicular fluid increased ($p < 0.01$) from 0 to 48 h after PGF (Table 3) and then declined ($p < 0.01$) between 48 and 72 h; onset of estrus occurred between 48 and 72 h. The change in E_2 of greatest magnitude occurred in follicular fluid of large E-A follicles; means were 106.0, 472.2, 888.3, and 390.0 ± 85.2 ng/ml E_2 at 0, 24, 48, and 72 h, respectively. Contrastingly, binding activity (Table 3) of IGFBP-2 in follicular fluid was lower at 24 h ($p < 0.05$) and 48 h ($p < 0.01$) after PGF, whereas binding by 29–27-kDa IGFBP was lower at 48 h ($p < 0.05$) and 72 h ($p < 0.01$) after PGF. Intensity of the IGFBP-3 and 22-kDa IGFBP bands was not influenced significantly ($p > 0.10$) by time.

Follicular fluid concentrations of IGF-I did not differ ($p > 0.10$) with time after the PGF injection but were higher ($p < 0.01$) in follicular fluid of large E-A follicles than in the other three stages of follicular development (Table 3). Medium follicles had higher ($p < 0.01$) IGF-I concentrations than either large E-I or small follicles. Overall, IGF-I was correlated positively ($p < 0.01$) with E_2 ($r = 0.39$) and A_4 ($r = 0.51$). The negative correlations between IGF-I and IGFBP-2, 29–27-kDa IGFBP, or 22- kDa IGFBP ($r = -0.18$, -0.17, or -0.15, respectively) were not significant ($p > 0.10$).

A_4 was lower ($p < 0.05$) in follicular fluid of large E-I follicles than in pools from small follicles (Table 3); follicular fluid from large E-A and medium follicles had intermediate concentrations of A_4. A_4 was correlated positively ($p < 0.01$) with E_2 ($r = 0.31$) and with binding activity of IGFBP-3 ($r = 0.47$); the positive correlations between A_4 and the other three IGFBP species were low ($p > 0.10$).

The only significant ($p < 0.05$) time × follicular stage interaction was for follicle diameter. The increase in diameter of large follicles (Table 2) from 0 to 72 h after PGF was significant ($p < 0.01$) for E-A (15.2 vs. 19.8 ± 1.0 mm) follicles but not ($p > 0.10$) for E-I (11.7 vs. 15.5 ± 2.1) follicles. Diameter of the medium and small follicle pools was unchanged. Follicular size had a positive influence ($p < 0.01$) on follicular fluid concentrations of E_2 ($r = 0.62$) but a negative influence ($p < 0.01$) on the binding activity of IGFBP-3, IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP ($r = -0.36$, -0.81, -0.73, and -0.81, respectively).

TABLE 3. Effect of time after PGF on ovarian follicular fluid E_2 and IGFBPs in control cows (experiment 2).[a]

Hours after PGF	No. follicles	E_2 (ng/ml)	IGFBP-3 (U/μl)	IGFBP-2 (U/μl)	29–27 kDa[b] (U/μl)	22 kDa (U/μl)
0	18	34.7 ± 58.6[c]	3.29 ± 0.27	2.18 ± 0.18[cf]	2.46 ± 0.32[cf]	0.52 ± 0.05
24	16	187.9 ± 69.4[def]	3.03 ± 0.32	1.51 ± 0.19[cdg]	1.68 ± 0.36[cd]	0.38 ± 0.05
48	17	374.5 ± 55.3[d]	2.85 ± 0.25	1.13 ± 0.17[d]	1.36 ± 0.30[cdg]	0.37 ± 0.04
72	17	123.7 ± 61.3[eg]	3.75 ± 0.28	1.53 ± 0.19[cdg]	0.92 ± 0.36[d]	0.33 ± 0.05

[a]Means ± SEM; means for each time interval were obtained from one measurement each for a large E-A follicle, a pool from medium follicles, and a pool from small follicles for each of 5 cows plus one to three values per time for large E-I follicles (see Table 2).
[b]Sum of IGFBP bands in the 29–27-kDa region.
[cde]Means without a common superscript differ by time ($p < 0.01$).
[fg]Means without a common superscript differ by time ($p < 0.05$).

TABLE 4. Effect of FSH and time after PGF on ovarian follicular fluid steriods, IGFBPs, and IGF-I (experiment 2).[a]

Hours after PGF	Status	N	Follicle diameter (mm)	E_2 (ng/ml)	IGFBP-3 (U/μl)	IGFBP-2 (U/μl)	29–27 kDa[b] (U/μl)	22 kDa (U/μl)	IGF-I (ng/ml)
Control									
0	E-active	5	15.2 ± 1.0	106.0 ± 59.3[c]	2.11 ± 0.21[k]	0.07 ± 0.19[cdg]	0.00 ± 0.19[c]	0.00 ± 0.04[c]	64.03 ± 3.67[k]
0	E-inactive	3	10.7 ± 1.3	1.8 ± 76.5[f]	4.52 ± 0.28	2.75 ± 0.24[ei]	3.08 ± 0.28[d]	0.51 ± 0.06[d]	46.33 ± 4.74
48	E-active	5	18.8 ± 1.0	888.3 ± 59.3[d]	2.04 ± 0.21	0.00 ± 0.19[c]	0.00 ± 0.19[c]	0.00 ± 0.04[c]	70.48 ± 3.67
48	E-inactive	2	15.0 ± 1.6	13.1 ± 93.7[f]	3.74 ± 0.34	1.23 ± 0.30[efj]	0.30 ± 0.10[c]	0.12 ± 0.02[c]	47.86 ± 5.80
FSH									
0	E-active	18	12.5 ± 0.5	491.3 ± 31.2[e]	2.26 ± 0.11	0.03 ± 0.10[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	64.37 ± 1.93
0	E-inactive	1	12.0	9.8[f]	1.96[c]	0.80[dfhj]	0.20[c]	0.09[c]	49.34
48	E-active	14	13.9 ± 0.6	122.8 ± 40.0[c]	2.15 ± 0.14	0.36 ± 0.13[dh]	0.02 ± 0.12[c]	0.01 ± 0.03[c]	73.14 ± 2.19
48	E-inactive	5	11.7 ± 1.0	11.6 ± 66.3[f]	4.10 ± 0.24	1.57 ± 0.21[f]	0.09 ± 0.21[c]	0.07 ± 0.05[c]	54.47 ± 3.67

[a]Means ± SEM; N = number of follicles collected from 5 control and 4 FSH-treated females per treatment × time group. Number of E-A and E-I follicles per control female was 1 and 0–1, respectively, and per FSH-treated female was 3–5 and 0–2, respectively.
[b]Sum of 29–27-kDa IGFBP bands.
[cdef]Means without a common superscript differ ($p < 0.01$).
[ghij]Means without a common superscript differ ($p < 0.05$).
[k]Concentrations of IGFBP-3 binding activity and IGF-I differed ($p < 0.01$) by follicular stage only.

Control vs. FSH. As no medium follicles and few small follicles were isolated from the ovaries of FSH cows, only data for E-A and E-I large follicles were included in the statistical analysis (Table 4). The FSH treatment stimulated follicular development in all eight cows treated as evidenced by the presence of 14–35 large follicles per pair of

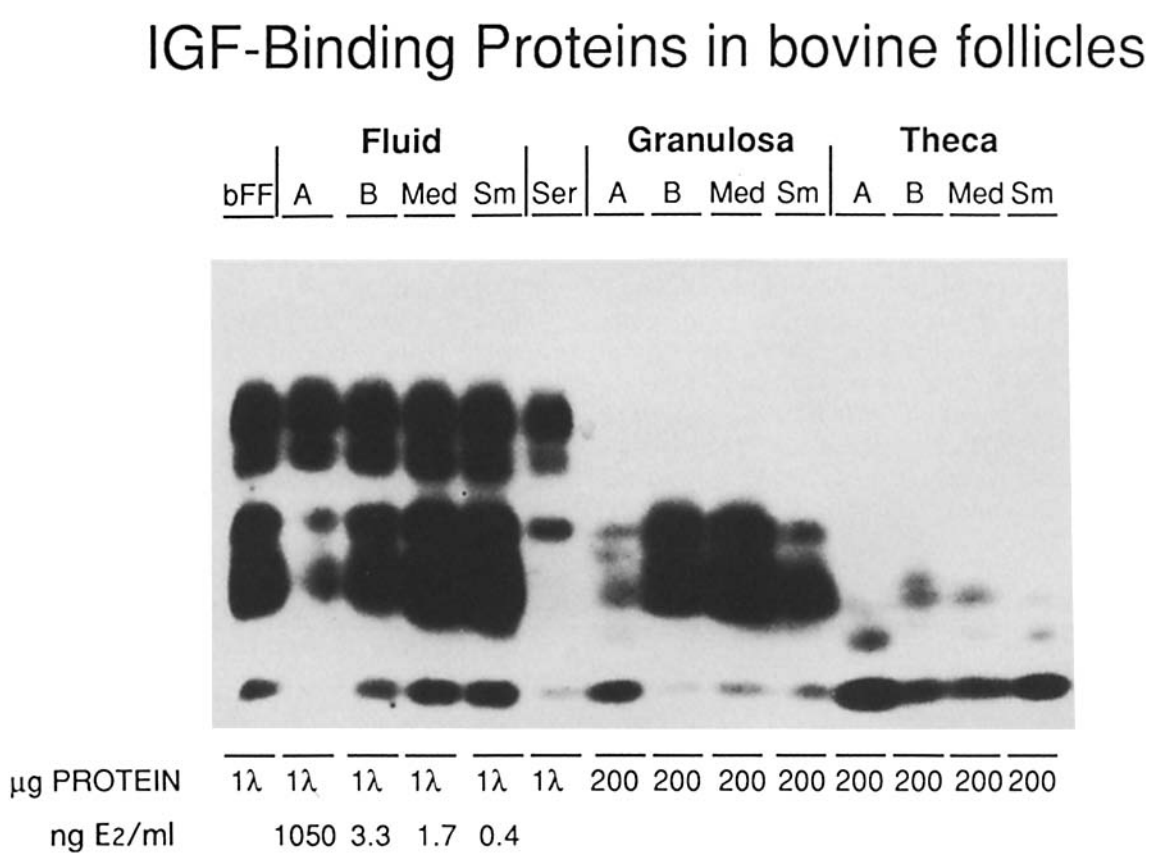

FIG. 6. Ligand blots of IGFBP binding activity in serum (1 μl), ovarian follicular fluid (1 μl), and homogenates (200 μg of total protein) of follicular granulosa and theca cells from the same animal. Ovarian samples were from large E-A (A) and E-I (B) follicles and from pools of medium (Med, 5–7 mm) and small (Sm, ≤ 5 mm) follicles.

ovaries. Of the 4–5 large follicles collected per FSH-treated cow, only 1 follicle was E-I at 0 h, while 2 follicles in two cows and 1 follicle in one cow were E-I at 48 h. Changes in follicular fluid E_2 concentrations with time after PGF differed between E-A large follicles from control vs. FSH cows and between E-A and E-I large follicles (follicular stage × treatment × time; $p < 0.05$). Concentrations of E_2 in E-A large follicles from controls increased ($p < 0.01$) between 0 and 48 h after PGF, while E_2 decreased ($p < 0.01$) in E-A large follicles from FSH cows between 0 and 48 h. In contrast, E_2 concentrations in follicular fluid of E-I large follicles of both control and FSH cows were lower ($p < 0.01$) than in fluid of E-A large follicles and were unaffected ($p > 0.1$) by time after PGF. Intensities of the IGFBP-2, 29–27-kDa IGFBP, and 22-kDa IGFBP bands (Table 4) were influenced by the interactions of follicular stage, treatment, and time (follicular stage × treatment × time; $p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively). Intensity of the IGFBP-2 band was greater ($p < 0.01$) in E-I than in E-A large follicles; intensity was unaffected by time after PGF in E-I follicles but increased ($p < 0.01$) between 0 and 48 h in E-A follicles of FSH cows. Binding activities for 29–27- and 22-kDa IGFBP were elevated ($p < 0.01$) in follicular fluid of E-I large follicles from controls at 0 h after PGF compared to E-I follicles from controls at 48 h or FSH cows at either 0 or 48 h, or to any of the E-A follicle groups. Binding activity of IGFBP-3 (Table 4) was greater ($p < 0.01$) in follicular fluid of E-I vs. E-A large follicles. Concentrations of IGF-I in follicular fluid did not differ ($p > 0.10$) between control and FSH cows (61.66 ± 6.43 vs. 65.60 ± 4.80 ng/ml) or between 0 and 48 h after PGF (61.74 ± 5.74 vs. 67.15 ± 5.00 ng/ml); but these concentrations were higher ($p < 0.01$) in E-A than in E-I large follicles (67.72 ± 4.53 vs. 50.19 ± 3.47 ng/ml). Overall, IGF-I was correlated positively with E_2 ($r = 0.46$; $p < 0.01$) and A_4 ($r = 0.33$; $p < 0.05$). Concentrations of A_4 in follicular fluid did not differ ($p > 0.1$) between E-A and E-I large follicles (38.5 ± 3.1 vs. 28.4 ± 6.0 ng/ml) or between control and FSH cows (41.1 ± 6.1 vs. 25.8 ± 6.0 ng/ml).

Cell extracts. Figure 6 is a ligand blot of IGFBP binding activity in ovarian follicular fluid (1 μl) and homogenates of washed granulosa and theca cells (200 μg protein) isolated separately from the same follicles. The 44- and 40-kDa bands present in follicular fluid were not detected in granulosa and theca cell homogenates.

DISCUSSION

Ligand blot analysis of bovine follicular fluid revealed quantitative and qualitative differences in IGFBP profiles due to size and physiological status of the follicle. Binding activities of IGFBP-2 and of the 29–27-kDa and 22-kDa IGFBPs were high in follicular fluid pools from small or medium follicles and in follicular fluid of individual large atretic (E-I) follicles, but were markedly reduced or nondetectable in follicular fluid of large preovulatory or dominant (E-A) follicles. Similar relationships between ovarian follicular fluid concentrations of IGFBPs and follicular functional status and/or size have been reported for swine [20), sheep [23], and humans [10]. While the identity of the 29–27- and 22-kDa IGFBPs was not determined, unpublished data from our laboratory and previous research [24] indicate that immunoreactivity for IGFBP-5 corresponds to binding in the 29-kDa region. In addition, previous results for human [10] and bovine [24] follicular fluid suggest that binding in the 22- and 27-kDa regions may represent different glycosylated forms of IGFBP-4. The proposed identity of these IGFBPs and results of our ligand blots are consistent with in situ hybridization analyses that revealed transcription of IGFBP-4 and -5 mRNA in granulosa cells of atretic, but not dominant, follicles from adult rat ovaries [12, 19]. In contrast to our results, the IGFBP-2 signal was found to be very strong in the thecal interstitial cells of all rat graafian follicles [12]. Transcription of IGFBP-2 mRNA was identified in both the granulosa and theca layers of porcine [25] and human [11] antral follicles, and transcription of IGFBP-4 and -5 mRNA was observed in the granulosa and stromal cells of human graafian follicles [11]; unfortunately, the functional status of the graafian follicles was not determined. Studies have not been conducted to identify IGFBP-1 and -6 in the bovine ovary. Because of the qualitative and quantitative similarities we are reporting in IGFBP profiles between follicular fluid and homogenated, washed granulosa cells from the same follicles, differences in follicular fluid concentrations of IGFBPs among follicles appeared to be related to predicted transcriptional/translational differences. However, the possibility of differences in IGFBP protease activity [13] among follicles cannot be excluded.

That we did not find IGFBP-3 in bovine granulosa and theca cell homogenates is consistent with the reported absence of transcription and translation of IGFBP-3 mRNA in rat [12], porcine [25], and human [11] ovarian follicles. Thus, we speculate that the IGFBP-3 measured in bovine follicular fluid in the present study was sequestered from the blood or from extrafollicular tissue [12, 24]. Sequestration, as opposed to secretion, of IGFBP-3 by antral follicles could account for the observation that follicular size and stage had little or no effect on follicular fluid IGFBP-3 binding activity. As expression of IGFBP-3 mRNA in porcine ovaries was found to be localized mainly in luteal tissue [25], production of IGFBP-3 [26–28] and IGFBP-3 mRNA [27] by cultured porcine granulosa cells may have resulted from luteinization-like changes (increased P_4 synthesis) associated with the long-term culture of granulosa cells.

While results from the present study demonstrate that follicular fluid profiles of IGFBPs vary with stage of ovarian follicular development in cattle, the physiological role(s) of these proteins remains to be determined. In a previous study, in vitro treatment of rat granulosa cells with purified porcine IGFBP-3 and -2 was found to inhibit E_2 and P_4 pro-

duction and FSH-stimulated granulosa cell proliferation by sequestering IGF-I or -II and preventing receptor binding of the IGFs [17]. In contrast, preincubation of bovine fibroblast with IGFBP-3 increased IGF-I binding to the membrane surface via increased membrane-associated IGFBP-3 rather than increased binding by type I IGF receptors [29]; the membrane-associated IGFBP-3 binding subsequently increased sensitivity and cellular responsiveness to IGF-I. Thus, several investigators have hypothesized that IGFBPs have a regulatory role in the selection of the dominant or ovulatory follicle(s) by modulating the biological activity of the IGFs within the follicle. The absence or low concentrations of binding activity for IGFBP-2 and smaller-molecular-mass IGFBPs in the follicular fluid of dominant or preovulatory follicles in the present study are consistent with such a hypothesis. The 150-kDa, GH-dependent, IGF-I-acid-labile subunit IGFBP-3 ternary complex has been linked to IGF-I/II transport and stability in body fluids. Thus, the elevated IGFBP-3 in follicular fluid regardless of follicular size or stage may serve to maintain a high level of exposure of follicular cells to IGF-I/II. Alternatively, as both IGF-I and IGFBPs (i.e., IGFBP-2, -4, and -5) are produced by the same follicular cell types, the IGFBPs may regulate the actions of IGF-I in an autocrine/paracrine fashion at the cellular level as opposed to endocrine regulation by follicular fluid IGFBP concentrations. Thus, IGFBP-3 concentrations in follicular fluid may have minimal effects on the responsiveness of follicular cells to IGFs. It is consistent with this proposed hypothesis that follicular fluid concentrations of IGF-I (experiment 1) were correlated negatively ($p < 0.01$) with the binding activity of IGFBP-2 and of 29–27-kDa and 22-kDa IGFBP ($r = -0.51$, -0.51, and -0.38, respectively) and positively ($p < 0.01$) with follicular fluid concentrations of E_2 ($r = 0.48$). The lack of a relationship between IGF-I and IGFBP-3 ($r = 0.04$) is consistent with our proposal that intrafollicular IGF-I is a composite of intrafollicular production and IGFBP-3 transport [6].

As reported previously [9], IGF-I concentrations were higher in follicular fluid of large E-A follicles than in either follicular fluid of large E-I follicles or pools of follicular fluid from medium or small follicles. Consequently, follicular fluid concentrations of IGF-I were correlated positively with E_2 ($r = 0.48$ and 0.37 for experiment 1 and 2, respectively). The ability of IGF-I to stimulate mitosis and steroidogenesis in follicular cells is well documented [1, 2, 6], whereas the origin of the IGF-I in bovine follicular fluid is less definitive. Expression of IGF-I mRNA by bovine granulosa cells [5, 6] and parallel fluctuations in serum and follicular fluid concentrations may serve as evidence for a combination of intra- and extrafollicular contributions. For example, administration of bovine somatotropin to cyclic beef cattle increased IGF-I and decreased IGFBP-2 in both serum and follicular fluid, whereas immunization against growth hormone (GH)-releasing factors decreased both serum and follicular fluid IGF-I and increased binding activity for IGFBP-2, -4, and -5 in follicular fluid [24]. However, the possibility of direct stimulation of intraovarian IGF-I production by GH cannot be excluded [6]. The physiological basis for the inverse relationship between IGF-I and the lower-molecular-mass IGFBPs in bovine follicular fluid remains to be determined, but it is inconsistent with in vitro [31] and in vivo [32] results reported for swine.

The predominant identifiable effect of the FSH treatment on ovarian function in the present study was increased numbers of large E-A or preovulatory follicles on the ovaries. Large E-A follicles from cows treated with FSH had higher follicular fluid concentrations of E_2 at 0 h after PGF, but lower concentrations at 48 h after PGF, than E-A follicles from controls. As the interval from PGF injection to estrus and preovulatory LH release is hastened by about 24 h in FSH-treated cattle [33, 34], it is assumed that some of the FSH cows (but not controls) had experienced a preovulatory LH release by 48 h after PGF and consequently the decrease in E_2. Likewise, although the binding activity of IGFBP-2 was very low in E-A follicles from FSH cows at both 0 and 48 h after PGF, it appeared to be increased at 48 h after PGF (Fig. 5; Table 4). Such an increase may suggest resumption of IGFBP production after exposure of the follicular cells to the preovulatory LH surge [20]. Collectively, differences in follicular fluid concentrations of E_2, IGF-I, and IGFBP binding activity between control and FSH cows appear to be associated with differences in stage of follicular development relative to time after the PGF injection, with follicles from FSH cows being more advanced.

The mechanism(s) by which FSH treatment results in superinduction of ovarian follicular development remains unclear. Such a mechanism may include FSH-mediated alterations in IGFBP production, or degradation within primary and/or secondary follicles, enabling IGF-I to influence the number of follicles recruited into the dominant status. Low dosages of FSH were found to increase total binding of IGF-I and binding activity of 28–29-kDa IGFBP in media conditioned by granulosa cells collected from ovaries of immature estrogen-primed rats; in contrast, high dosages of FSH inhibited total IGF-I binding and binding activity of 28–29-kDa IGFBP [35, 36]. Binding of IGF-I to the 23-kDa IGFBP increased initially and then decreased in response to FSH. Similarly, FSH, but not E_2, inhibited production of IGFBP-3 and IGFBP-2 by luteinized porcine granulosa cells in culture [26, 28]. Alternatively, such reductions in IGFBPs may result from degradation of IGFBP-5 by FSH-inducible protease [37], or from IGF-I- or IGF-II-promoted proteolytic degradation of IGFBP-4 [38] into fragments that did not bind IGFs.

In conclusion, the reduction in binding activity of the IGFBPs in E-A preovulatory follicles observed in the present study is consistent with the proposed autocrine/paracrine regulatory role of IGFBP-2, -4, and -5 on the biological activity of IGF-I and/or IGF-II and their effects on mitosis and steroidogenesis in ovarian follicular cells (i.e., follicu-

logenesis). Thus, fluctuations in endogenous FSH release during the bovine estrous cycle may modulate follicular development and atresia by attenuating the release or availability of IGFBPs. The IGFBPs in turn modulate the access of endogenous IGF-I to its cognate cell surface receptors and its hormonal action.

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