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Levels of Insulin-Like Growth Factor (IGF) Binding Proteins, Luteinizing Hormone and IGF-I Receptors, and Steroids in Dominant Follicles during the First Follicular Wave in Cattle Exhibiting Regular Estrous Cycles

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ABSTRACT

Objectives of this study were to determine if concentrations of steroids, insulin-like growth factor -I (IGF-I), and IGF binding proteins (IGFBP) in follicular fluid and numbers of LH and IGF-I receptors change during growth of the dominant follicle. Ovarian follicular development was monitored daily via ultrasound in lactating Holstein cows. Animals underwent bilateral ovariectomy when the dominant follicle was first identified (days 4–6; estrus = day 0; early; n = 5) or when it stopped growing (days 8–12; late; n = 8). All follicles were classified as dominant (DF), large (LG; ≥ 6 mm in diameter, excluding DF) or small (SM; < 6 mm), follicular fluid was aspirated, and theca and granulosa cells were collected. Levels of IGFBP-2, assessed via ligand blotting, were greater ($P < 0.05$) in LG and SM follicles compared with DF in early cows. Levels of IGFBP-3 in follicular fluid were unaffected by follicle class. Numbers of specific ^{125}I-hCG/LH binding sites in thecal cells were greater ($P < 0.01$) in DF compared with LG and SM follicles of both early and late cows. Numbers of specific ^{125}I-hCG/LH binding sites in granulosa cells were similar for follicle sizes in early cows, but, in late cows, were greater ($P < 0.01$) in DF compared with SM follicles and were severalfold greater ($P < 0.01$) in late DF compared with early DF. Numbers of receptors for IGF-I in thecal cells were 2-fold greater ($P < 0.05$) in DF and LG compared with SM in late cows. Numbers of IGF-I receptors in granulosa cells were unaffected by size or growth of follicles, but were severalfold greater than in theca cells. Concentrations of estradiol were severalfold greater ($P < 0.01$) in DF compared with LG and SM in both early and late cows. Concentrations of androstenedione in early cows were greater ($P < 0.05$) in DF and SM compared with LG follicles. Concentrations of progesterone and IGF-I did not differ ($P > 0.10$) among follicle classes, but both were greater ($P < 0.10$) in late LG compared with early LG follicles. Concentrations of IGF-II in follicular fluid did not differ ($P > 0.10$) between early and late cows but were greater ($P < 0.10$) in SM than DF or LG follicles. We conclude that low amounts of IGFBP-2 and increased thecal binding sites for hCG/LH appear to be related to establishment of the dominant follicle during the first follicular wave in cattle exhibiting regular estrous cycles during late lactation. (*Endocrinology* **137:** 2842–2850, 1996)

IN CATTLE, follicular growth during the estrous cycle has been associated with usually two to three follicular waves, with each wave consisting of a dominant follicle and associated subordinate follicles, based on studies using ultrasonography (1, 2). Recent studies have focused on the regulation of the first-wave (nonovulatory) dominant follicles. Guilbault *et al.* (3) reported that growing dominant follicles in the first follicular wave of an estrous cycle had high estradiol:androstenedione ratios, whereas the regressing dominant follicles had very low ratios. This agrees with Ireland and Roche (4), who demonstrated that estrogen-active follicles on days 5 and 7 of a bovine estrous cycle had increased numbers of granulosa cells and ability of thecal cells to bind LH and few histological signs of atresia compared with estrogen-active follicles on day 3 of an estrous cycle. The endocrine factors responsible for these changes in follicular function are uncertain. One such potential intraovarian regulator is insulin-like growth factor-I (IGF-I), which has been shown to be produced locally within the ovary (5, 6), and concentrations of which increase with follicle diameter in some (7, 8) but not all (9) studies. In preantral follicles, Wandji *et al.* (10) demonstrated that there is a dearth of gonadotropin binding sites and almost no specific IGF-I binding sites, but after formation of the antrum, numbers of gonadotropin and IGF-I binding sites increase. Part of the actions of IGF-I on follicular development involve stimulation of granulosa and thecal cell proliferation and steroidogenesis (5, 6, 11) as well as preventing ovarian follicular cell apoptosis (12).

It is thought that the availability of IGF-I for use by granulosa and thecal cells is affected by various IGF binding proteins (IGFBP) (6, 13–15). Generally, IGFBPs are inhibitory to the actions of IGF-I (6, 16, 17). However, IGFBP-3 possibly is involved in maintaining intrafollicular IGF-I concentrations, whereas IGFBP-2 and a 29-kDa and 22-kDa IGFBP are

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negatively correlated with follicular fluid concentrations of IGF-I (13). Therefore, in growing antral follicles with decreased amounts of IGFBP-2 and other low molecular weight IGFBPs, more IGF-I should be available for binding with receptors. Also, granulosa cells from large follicles have been shown to have increased numbers of IGF-I receptors as compared with small follicles (18). We have demonstrated that the synergistic actions of IGF-I on LH-induced steroidogenesis by bovine thecal cells are mediated, in part, through an IGF-I-induced increase in the number of LH receptors (11). As mentioned, previous research (4) has shown that increased hCG/LH binding sites in theca cells were associated with increased diameters of estrogen-active follicles during days 3 to 7 of the bovine estrous cycle, whereas FSH binding sites in granulosa cells of estrogen-active follicles did not change during the same interval (4). Also, estrogen-inactive follicles from days 9–13 were smaller with less granulosa cells and fewer thecal and granulosa LH/hCG binding sites (4). These biochemical/physiological changes for estrogen-active follicles also have been demonstrated in ovulatory follicles (19).

The increase in IGF-I-binding sites followed by the increase in hCG/LH binding sites in developing bovine follicles (9) indicate a possible role for IGF-I in the regulation of growth of follicles. However, research examining differences in IGF-I binding sites and IGFBPs between growing and regressing nonovulatory dominant follicles has not been done. Therefore, our objective of this study was to determine if concentrations of steroids, IGF-I, IGF-II, and IGFBPs, and numbers of receptors for IGF-I and LH change during the transition from a growing phase to a nongrowing (static) phase of first-wave dominant follicles in cattle exhibiting regular estrous cycles during late lactation.

Materials and Methods

Animals and experimental protocol

Eighteen lactating Holstein cows were used for this study. These cows were identified to be culled for nonreproductive reasons from the University herd in an attempt to reduce the total herd size from 180 to 130 cows. Cows were milked twice daily (0300 and 1500 h), housed on pasture, and group-fed a total mixed ration consisting of sorghum silage, alfalfa hay, whole cottonseed, and concentrate *ad libitum*. Estrus was monitored twice daily. Three cows failed to exhibit estrus and two developed follicular cysts (*i.e.* follicles > 25 mm existing for > 4 days) and were eliminated from the experiment. Estrous cycle interval, body weight at the termination of the experiment, days postpartum, daily milk production, and average number of lactations for the remaining 13 cows were recorded.

Estrous cycles were synchronized using two injections (im) of prostaglandin $F_{2\alpha}$ (Lutalyse®, 25 mg) 11 days apart. Starting at estrus, follicular development was monitored daily for an entire estrous cycle via ultrasonography, using an Aloka 500V with a 7.5 MHz probe. Number of follicular waves were determined for each cow. All cows had either 2 or 3 follicular waves. At the subsequent estrus (day 0), cows were assigned to be ovariectomized either at day 4 to 6 (early growing phase of the first dominant follicle; n = 4 cows with 2 follicular waves and 1 cow with 3 follicular waves from the previous estrous cycle) or days 8–12 (late growing phase of the first dominant follicle; n = 6 cows with 2 follicular waves and 2 cows with 3 follicular waves from the previous estrous cycle). All of the 13 cows had a single follicle ovulate after the subsequent estrus; however, one cow in each group had delivered twin calves at their previous calving. Blood samples were obtained daily via jugular venipuncture from day 0 until ovariectomy. Ultrasound examinations were performed daily, continuing from the previous estrous cycle. As soon as the first dominant follicle could be identified from its subordinates (*i.e.* the first day the largest growing follicle diameter was ≥ 2 mm from any other follicle), cows were either bilaterally ovariectomized or follicular development was monitored until the first dominant follicle slowed in its growth or stopped growing (*i.e.* the first day the dominant follicle grew ≤ 1 mm from the previous day). Both ovaries from each cow were removed via lateral incision through the left paralumbar fossa area after local anesthesia (2% lidocaine; 60 to 80 ml sc and im). After each ovariectomy, ovaries were identified as right and left, put on ice, and transported to the laboratory where diameters of all follicles ≥ 6 mm in diameter were recorded, the numbers of all follicles ≥ 1 mm in diameter on the ovarian surface determined, and ovarian tissue and fluid collected. One dominant follicle from a cow ovariectomized late accidentally ruptured at the time of ovariectomy, and thus its follicular fluid was not collected. The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Collection of follicular cells and fluid and corpora lutea

Follicular fluid from individual follicles ≥ 6 mm in diameter (classified as dominant or large) was aspirated and centrifuged to obtain granulosa cells (20), and follicular fluid from follicles < 6 mm in diameter (small) was pooled within ovaries and then centrifuged to obtain granulosa cells. After centrifugation, follicular fluid was aspirated and stored in another tube at -20 C. After aspiration of follicular fluid, each follicle ≥ 6 mm in diameter was bisected, and the inner wall was scraped and rinsed with Ham's F-12 to remove any remaining granulosa cells. Granulosa cells separated from follicular fluid of large follicles were combined with granulosa cells collected after scraping. The theca interna layer was removed by blunt dissection. For small follicles, the follicular wall was removed by blunt dissection after bisection of each follicle; granulosa cells were not separated from the theca interna. Granulosa cells separated from follicular fluid of small follicles were combined from both ovaries. Each corpora lutea was dissected free of ovarian stroma, weighed, and quartered. All tissue was stored frozen at -70 C in PBS containing 20% glycerol.

RIAs

Concentrations of androstenedione in follicular fluid were determined in one assay using a solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA) as previously described (11). The intraassay CV was 2.0%. Increasing volume (4, 8, 12.5, 25, 50, 75, and 100 μl) of diluted (1:10) follicular fluid displaced [^{125}I]-androstenedione from the antiserum to produce a binding curve parallel to the standard curve. Recovery of androstenedione added to 1.0 μl of follicular fluid averaged 101 ± 5% (n = 6 determinations) and assay sensitivity was 2.5 pg/tube. Concentrations of estradiol in follicular fluid were determined by RIA as previously described (8). The intra- and interassay CV were 7.0 and 14.0%, respectively. Concentrations of progesterone in plasma were determined in one assay using a solid-phase RIA kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The intraassay CV was 11.1%. Increasing volume (10, 25, 50, 75, 100, and 200 μl) of plasma displaced [^{125}I]-progesterone from the antiserum to produce a binding curve parallel to the standard curve. Recovery of progesterone added to 100 μl of plasma averaged 99.7 ± 5.3% (n = 9 determinations), and assay sensitivity was 0.05 ng/ml. Concentrations of progesterone in follicular fluid were determined by RIA in one assay as previously described (8). The intraassay CV was 14.0%. Concentrations of IGF-I in plasma and follicular fluid were determined by RIA as previously described (21). The intra- and interassay CV were 12 and 15%, respectively. Concentrations of IGF-II in plasma and follicular fluid were determined by RIA in one assay as previously described (22). The intraassay CV was 2.6%.

Binding of ^{125}I-IGF-I and ^{125}I-hCG to thecal and granulosa cells

Numbers of binding sites for IGF-I (11, 18) and hCG/LH (23, 24) in bovine thecal and granulosa cells were determined as previously described. Briefly, on the day of assay, ovarian tissue samples were thawed

and homogenized separately over ice using a Teflon to glass homogenizer and then filtered through two layers of cheesecloth. After filtering, homogenates were rinsed twice in PBS. After each rinse with PBS, homogenates were centrifuged at 2,200 × *g* for 5 min at 4 C. After the final rinse, wet weight of each homogenate was recorded and PBS added for a final concentration of 0.1 to 1.5 mg/10 μl. Amount of DNA in each homogenate was determined (25). Specific binding of ^{125}I-IGF-I and ^{125}I-hCG was expressed as counts per minute per microgram of DNA. To determine specific binding of each labeled hormone in granulosa, theca, and follicular wall tissue, 50 μl aliquots were pipetted into polypropylene tubes previously coated with PBS-5% BSA and incubated with saturating amounts of ^{125}I-IGF-I (130,000 cpm/tube) for 16 h at 4 C in the presence or absence of excess unlabeled IGF-I (250 ng/tube) or ^{125}I-hCG (170,000 cpm/tube) for 24 h at 25 C in a shaker in the presence or absence of unlabelled bLH (5.0 μg/tube). After incubation, samples were rinsed twice with cold PBS. After each rinse, samples were centrifuged at 2200 × *g* for 15 min at 4 C, the supernatant aspirated, and the precipitates counted in an automated gamma counter (counting efficiency = 75%).

Ligand blots

Follicular fluid and plasma IGFBPs were analyzed by one-dimensional SDS-PAGE, as previously described (13). Briefly, samples were heat denatured and then separated on a 12% polyacrylamide gel via electrophoresis. After separation, proteins in gels were electrophoretically transferred to nitrocellulose, and ligand-blotted overnight with ^{125}I-IGF-I. After washing and then exposure to x-ray film at -70 C for 48 h, band intensity on autoradiographs was determined using scanning densitometry.

Statistical analyses

The percentage of cows with two follicular waves in the group ovariectomized early (80%) as compared with those ovariectomized late (75%) was similar ($P > 0.10$, Chi-square), and thus data for cows with two and three follicular waves were combined within time of ovariectomy for analysis. Follicular fluid hormonal and follicular cell receptor data were analyzed using the least squares ANOVA by the GLM procedure of SAS (26). Data with heterogeneous variances (follicular fluid estradiol, progesterone, and androstenedione) were analyzed after transformation to natural log (x + 1). Main effects, consisting of time of ovariectomy (early and late) and follicle category (small, large, and dominant follicle) and their various interactions were analyzed. Daily plasma hormonal data (progesterone and IGF-I) were analyzed using a split plot ANOVA for repeated measures over time; effects of time of ovariectomy were tested using cow within time group as the error term and effects of day and time group by day were tested using the residual error term. For estrous cycle interval, body weight, days postpartum, milk weight, lactation number, numbers of follicles, and ovarian and CL weights, the main effect was time of ovariectomy. For IGFBP data, main effects included time of ovariectomy and follicular category, with gel number included in the model as a blocking factor. Specific differences between means were determined using Fisher's protected least significant difference procedure (27) if significant main effects were detected.

Results

Physiological characteristics for cows ovariectomized early *vs.* late did not differ ($P > 0.10$): estrous interval, final body weight, days postpartum, daily milk production and lactation number averaged (± SE) 23.2 ± 1.4 days, 658 ± 43 kg, 319 ± 59 days, 17.5 ± 4.0 kg/day and 2.8 ± .7, respectively for cows ovariectomized early and 21.1 ± 1.1 days, 707 ± 34 kg, 336 ± 47 days, 16.0 ± 3.1 kg/day and 3.3 ± .5, respectively, for cows ovariectomized late. Ovarian characteristics are listed in Table 1. Total ovarian weight, numbers of small and large follicles and average diameter of large follicles (excluding dominant follicles) did not differ ($P > 0.10$) between early and late cows. However, day of cycle, CL weight and average diameter of dominant follicles were greater in late than early cows (Table 1).

Follicle category affected ($P < 0.01$) follicular fluid concentrations of estradiol, whereas day of ovariectomy and follicle category by day of ovariectomy interaction were not significant. Concentrations of estradiol in follicular fluid were greatest ($P < 0.01$) in dominant compared with large and small follicles in cows ovariectomized both early and late during the first follicular wave (Table 2). For both early and late groups, large and small follicles had similar ($P > 0.10$) concentrations of estradiol.

Follicle category and its interaction with day of ovariectomy had no effect ($P > 0.10$), whereas day of ovariectomy tended ($P < 0.10$) to influence concentrations of progesterone in follicular fluid (Table 2). Averaged across follicle category, concentrations of progesterone tended to be greater ($P < 0.10$) in follicles from cows ovariectomized late (116 ± 18 ng/ml) compared with cows ovariectomized early (65 ± 21 ng/ml) during the follicular wave. Ratios of estradiol: progesterone were affected ($P < 0.05$) by day of ovariectomy, follicle category and their interaction. Ratios of estradiol: progesterone revealed that all dominant follicles were estrogen active but less so late during the follicular wave (Table 2).

Follicle category affected ($P < 0.05$) follicular fluid androstenedione concentrations, whereas day of ovariectomy and its interaction with follicle category was without effect ($P > 0.10$). Averaged across early and late groups, concentrations of androstenedione in follicular fluid were 5.6-fold greater ($P < 0.05$) in dominant follicles compared with large follicles and 2.4-fold greater ($P < 0.05$) in small follicles compared with large follicles (Table 2). Dominant and small follicles from cows ovariectomized early or late during the first follicular wave had similar concentrations of androstenedione (Table 2). The estradiol: androstenedione ratio was affected by follicle category ($P < 0.05$) but not ($P > 0.10$) day of ovariectomy or their interaction (Table 2). Averaged for cows ovariectomized early and late, estradiol:androstenedione ratios averaged 9.4, 2.4, and 0.05 for dominant, large, and small follicles, respectively ($P < 0.05$).

TABLE 1. Average day of estrous cycle, ovarian and CL weight, diameter of dominant and large (≥6 mm) follicles, and numbers of small (<6.0 mm) and large follicles for Holstein cows ovariectomized early or late during the first follicular wave

Time of ovariectomy	N	Day of cycle	Ovarian weight, g	CL weight, g	Diameter of dominant follicles, mm	Diameter of large follicles[a], mm	No. of large follicles	No. of small follicles
Early	5	5.0	21.2	2.41	11.9	8.5	4.6	39.8
Late	8	9.9[b]	24.8	4.57[b]	17.2[b]	8.8	2.8	46.6
SE		0.5	2.0	0.68	0.9	0.9	0.8	11.8

[a] This value includes all large (≥6 mm) follicles except for dominant follicles.
[b] $P < .05$ *vs.* early group.

TABLE 2. Concentrations of E_2, P_4, A_4, and IGF-I in bovine follicular fluid from dominant, large (≥6 mm in diameter excluding dominant), and small (<6 mm in diameter) follicles from cows ovariectomized early or late during the first follicular wave of an estrous cycle

Variable	Time of ovariectomy	Follicle category[a]			Pooled SE
		Dominant	Large	Small	
E_2, ng/ml					
	Early	202.7[b]	12.4[c]	1.8[c]	21.6
	Late	107.5[b]	26.6[c]	2.6[c]	18.3
P_4, ng/ml					
	Early	53.4	60.3	81.3	35.1
	Late	60.3	155.5	130.9	30.2
A_4, ng/ml					
	Early	216.5[b]	25.2[d]	70.4[b,c]	24.7
	Late	65.5[b,c,d]	25.6[c,d]	45.8[b,c,d]	21.2
E_2:P_4 ratio					
	Early	5.87[b]	0.25[c,d]	0.04[d]	0.62
	Late	1.86[c]	0.50[c,d]	0.03[d]	0.54
E_2:A_4 ratio					
	Early	13.16[b]	4.08[c]	0.06[c]	3.58
	Late	5.64[b,c]	0.75[c]	0.05[c]	3.07
IGF-I, ng/ml					
	Early	89.1[e,f]	73.7[e]	99.4[e,f]	15.0
	Late	89.0[e,f]	120.4[f]	120.2[f]	12.9
IGF-II, ng/ml					
	Early	195.5	176.6	215.7	25.6
	Late	170.4	200.1	237.7	20.5

[a] Numbers of samples for dominant, large and small categories were 5, 18, and 8, respectively, for the early group and 7, 15, and 16, respectively, for the late group.

[b,c,d] Within a variable, means without a common superscript differ ($P < 0.05$).

[e,f,g] Means without a common superscript differ ($P < 0.10$).

Concentrations of IGF-I in follicular fluid did not differ ($P > 0.10$) among follicle categories. However, cows ovariectomized early during the first follicular wave tended to have lower ($P < 0.10$) IGF-I concentrations in large follicles than cows ovariectomized late (Table 2). No significant time of ovariectomy by follicle category interaction was detected. Although diameter of large follicles tended to correlate with concentrations of follicular fluid IGF-I ($r = 0.27$, $P < 0.10$), the percentage of follicular fluid IGF-I to plasma IGF-I did not differ ($P > 0.10$) among follicle categories and averaged 79 ± 7%.

There were no differences ($P > 0.10$) in progesterone and IGF-I concentrations during the first 5 days of the estrous cycle between cows ovariectomized early or late during the first follicular wave (data not shown). Also, no significant interaction between day of cycle and ovariectomy group existed for plasma progesterone and IGF-I concentrations. Concentrations of IGF-I in plasma averaged 89 ± 9 ng/ml and did not ($P > 0.10$) change between day 0 and 5, whereas plasma progesterone increased ($P < 0.05$) from 0.14 ng/ml on day 0 to 0.78 ± 0.08 ng/ml on day 5 of the estrous cycle.

Concentrations of IGF-II in follicular fluid was not affected ($P > 0.10$) by day of ovariectomy or its interaction with follicle category. However, follicle category tended ($P < 0.10$) to influence follicular fluid IGF-II concentrations such that small follicles had greater IGF-II concentrations (226.7 ± 15.3 ng/ml) than did dominant (182.9 ± 18.0 ng/ml) or large (188.3 ± 16.4 ng/ml) follicles (Table 2). Plasma concentrations of IGF-II on the day of ovariectomy averaged 110 ± 10 ng/ml and 110 ± 8 ng/ml for early and late cows, respectively, and did not differ ($P > 0.10$). The percentage of follicular fluid IGF-II to plasma IGF-II was affected ($P < 0.05$) by follicle category but not day of ovariectomy or their interaction (data not shown). Averaged across early and late cows, small follicles had a greater ($P < 0.05$) percentage of follicular fluid IGF-II to plasma IGF-II (196 ± 11%) than dominant (162 ± 13%) or large (155 ± 12%) follicles.

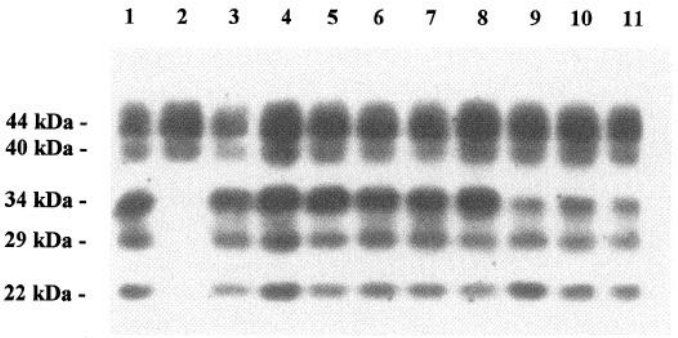

FIG. 1. A representative ligand blot analysis of IGFBPs in follicular fluid (FFL) of cows ovariectomized early or late during the first follicular wave of an estrous cycle. Lane 1, Pool of bovine FFL; lanes 2–8, FFL samples of a cow ovariectomized early (lane 2 = dominant follicle; lanes 3, 5, 6 and 7, large subordinate follicles; lanes 4 and 8, small FFL pools); lanes 9–11, FFL samples of a cow ovariectomized late (lane 9 = large subordinate follicle; lane 10 and 11, small FFL pools).

Ligand blotting revealed at least four forms of IGFBP binding activity in bovine follicular fluid. These included a doublet at 40–44 kDa and singlets at 34-, 29-, and 22-kDa (Fig. 1). The doublet at 40–44 kDa and the singlet at 34-kDa were identified as IGFBP-3 and IGFBP-2, respectively (see Ref. 13). The singlets at 29 and 22 kDa could not be identified. Binding activity of IGFBP-3 was unaffected ($P > 0.10$) by category of follicles (small, large, or dominant), day of ovariectomy or their interaction (Fig. 2A). In contrast, binding activity of IGFBP-2 was affected by follicle category ($P < 0.01$) and the interaction between follicle category and day ($P < 0.01$), but not day of ovariectomy ($P > 0.10$). In comparison, binding activity of the 22-kDa IGFBP and 29-kDa IGFBP was affected ($P < 0.01$) by follicle category but not affected ($P > 0.10$) by day of ovariectomy or their interaction. In cows ovariectomized early, binding activity of IGFBP-2 in large and small follicles was severalfold greater ($P < 0.05$) than in dominant follicles (Fig. 2B). Also, binding activity of IGFBP-2 in dominant follicles increased, whereas IGFBP-2 activity decreased in large and small follicles from early to late in the follicular wave such that IGFBP-2 activity in dominant and large follicles did not differ late in the follicular wave (Fig. 2B). Binding activity of the 29-kDa and 22-kDa proteins in follicles of cows ovariectomized early was greater ($P < 0.05$) in small and large follicles than dominant follicles; a difference in these two IGFBPs between dominant and large follicles did not exist in cows ovariectomized late in the first follicular wave (Fig. 2C). Binding activity of the 22-kDa IGFBP was greater ($P < 0.05$) in small follicles than large follicles in cows ovariectomized early and late (Fig. 2D). Total IGFBP activity (*i.e.* activity of all four IGFBP added) was affected by follicle category ($P < 0.05$) but not day of ovariectomy or their interaction; total IGFBP activity averaged 5.5, 12.9, and 17.3 ± 2.2 arbitrary units for dominant, large and small follicles,

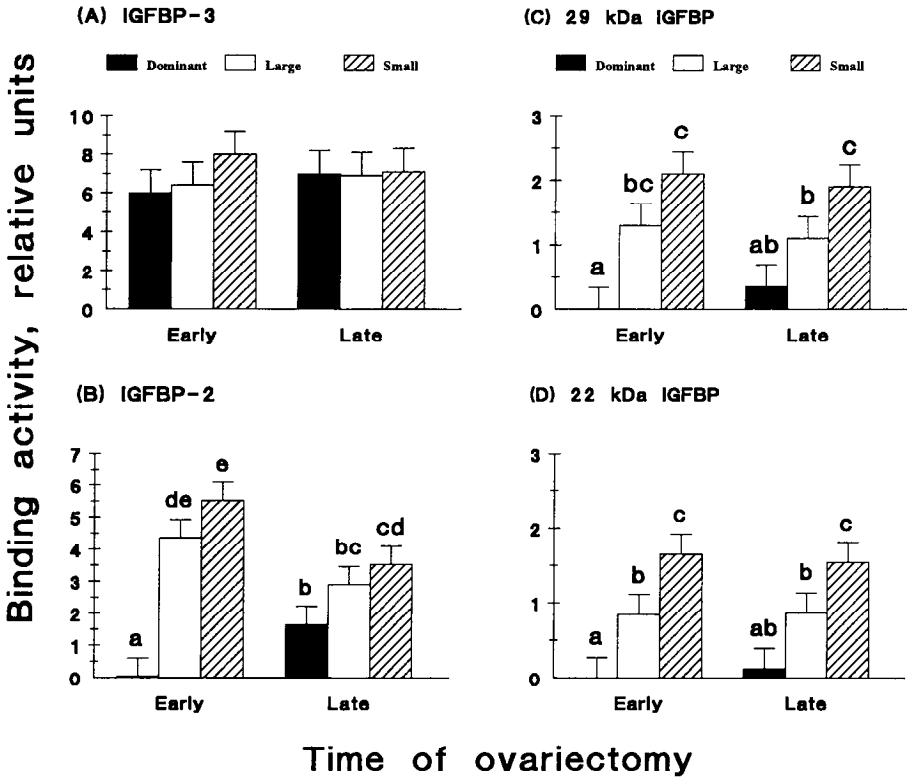

FIG. 2. Binding activity (in relative units) of IGFBP-3 (A) and IGFBP-2 (B) and the 29-kDa (C) and 22-kDa (D) IGFBPs in follicular fluid collected from dominant, large (≥ 6 mm in diameter excluding dominant), and small (< 6 mm in diameter) follicles from cows ovariectomized early or late during the first follicular wave of an estrous cycle. [a,b,c,d]Within a panel, means without a common letter differ ($P < 0.05$).

respectively, from cows ovariectomized early and 9.1, 11.7, and 14.0 ± 2.0 arbitrary units, respectively, from cows ovariectomized late. Binding activities of IGFBP-3, IGFBP-2, and the 29-kDa and 22-kDa IGFBPs in plasma at ovariectomy were not affected ($P > 0.10$) by day of ovariectomy (data not shown). Averaged across day of ovariectomy, plasma binding activity of IGFBP-3, IGFBP-2, the 29-kDa IGFBP and the 22-kDa IGFBP was 9.3 ± .6, 4.1 ± .3, 3.1 ± .2, and 1.6 ± .1 arbitrary units, respectively.

Day of ovariectomy and its interaction with follicle category had no effect ($P > 0.10$), whereas follicle category influenced ($P < 0.01$) specific binding of ^{125}I-IGF-I to thecal cells (Fig. 3A). Specific binding of ^{125}I-IGF-I to thecal cells was similar ($P > 0.10$) between dominant and large follicles from cows ovariectomized both early and late (Fig. 3A). Also, specific binding of ^{125}I-IGF-I to thecal cells did not differ ($P > 0.10$) between small and large or dominant follicles from cows ovariectomized early, but was lower ($P < 0.05$) in small than in dominant and large follicles of cows ovariectomized late (Fig. 3A).

Specific binding of ^{125}I-IGF-I to granulosa cells was unaffected ($P > 0.10$) by day of ovariectomy, follicle category, or their interaction (Fig. 3B). Granulosa cells contained 3- to 5-fold greater specific binding of ^{125}I-IGF-I than thecal cells (Fig. 3).

Similar to thecal IGF-I binding sites, day of ovariectomy, and its interaction with follicle category had no effect ($P > 0.10$), whereas follicle category influenced ($P < 0.01$) specific binding of ^{125}I-hCG to thecal cells. Specific binding of ^{125}I-hCG to thecal cells was 1.9- and 4.0-fold higher ($P < 0.01$) in dominant follicles compared with large and small follicles, respectively, in cows ovariectomized early in the growing

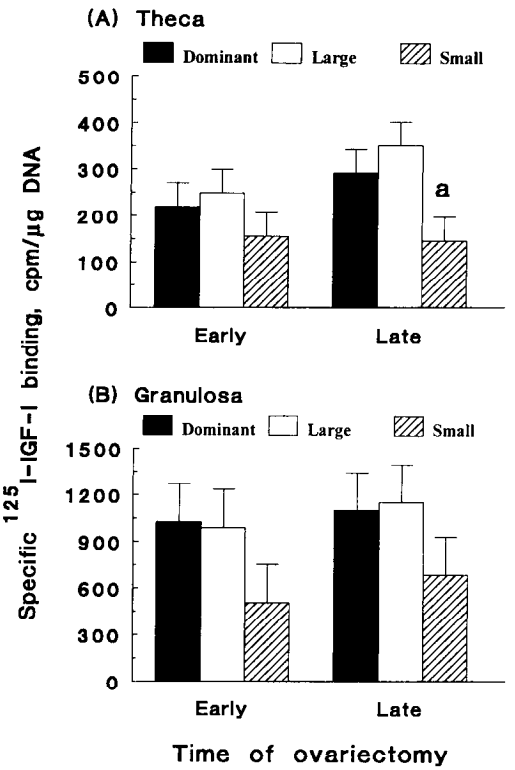

FIG. 3. Specific binding of ^{125}I-IGF-I to thecal (A) and granulosa (B) cells collected from dominant, large (≥ 6 mm in diameter excluding dominant), and small (< 6 mm in diameter) follicles from cows ovariectomized early or late during the first follicular wave of an estrous cycle. [a]Late small differs from late dominant and large ($P < 0.05$).

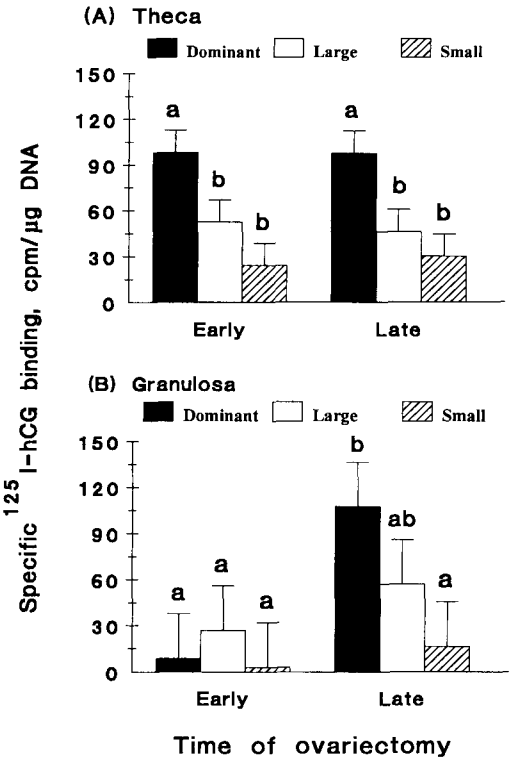

FIG. 4. Specific binding of ^{125}I-hCG to thecal (A) and granulosa (B) cells collected from dominant, large (≥ 6 mm in diameter excluding dominant), and small (< 6 mm in diameter) follicles from cows ovariectomized early or late during the first follicular wave of an estrous cycle. [a,b]Within a panel, means without a common letter differ ($P < 0.05$).

phase, and 2.1- and 3.2-fold higher ($P < 0.05$) in dominant follicles compared with large and small follicles, respectively, in cows ovariectomized late in the growing phase (Fig. 4A). Thecal cells from large and small follicles in both groups had similar ($P > 0.10$) specific binding of ^{125}I-hCG.

Day of ovariectomy influenced ($P < 0.05$) specific binding of ^{125}I-hCG to granulosa cells, whereas follicle category and its interaction with day had no effect ($P > 0.10$). Specific binding of ^{125}I-hCG to granulosa cells did not differ between dominant, large, or small follicles from cows that were ovariectomized early (Fig. 4B). However, in cows that were ovariectomized late, specific binding of ^{125}I-hCG to granulosa cells was 6.5-fold greater ($P < 0.05$) in dominant follicles than in small follicles. Also, specific binding of ^{125}I-hCG was 12.5-fold greater ($P < 0.05$) in granulosa cells of dominant follicles from cows ovariectomized late compared with dominant follicles in the early growing phase. Thecal cells contained severalfold greater specific binding of ^{125}I-hCG than granulosa cells during the early growing phase, but these differences did not exist during the late growing phase (Fig. 4).

Discussion

Results of the present study indicate that 1) dominant follicles early in their development had greater concentrations of estradiol and androstenedione and lower IGFBP-2, 29-kDa, and 22-kDa IGFBP activity in follicular fluid than did subordinate large and (or) small follicles; 2) dominant follicles had concentrations of progesterone, IGF-I, IGF-II and IGFBP-3 activity similar to those found in subordinate large follicles, regardless of their stage of development; 3) specific binding of ^{125}I-IGF-I to granulosa and thecal cells did not differ between dominant follicles and subordinate large follicles both early and late in their development; 4) specific binding of ^{125}I-hCG to thecal cells was greater in dominant follicles than subordinate large and small follicles both early and late in their development; and 5) specific binding of ^{125}I-hCG to granulosa cells was greater in dominant follicles than subordinate large and small follicles late but not early in their development.

Estradiol concentrations were greatest in dominant follicles compared with subordinates regardless if they were in the early or late growing phase, which agrees with earlier reports in cattle (1, 3, 4, 19, 28–31). As the first dominant follicle regresses, concentrations of estradiol in its follicular fluid decrease (3, 28) and are associated with a decrease in follicular P450 aromatase mRNA (29). The loss in the ability of estrogen-active follicles to produce estradiol during the first two weeks of an estrous cycle has been associated with decreased numbers of granulosa cells and reduced numbers of LH receptors in granulosa and thecal cells (4). Levels of androstenedione also were greater in dominant follicles early in their growth phase compared with their subordinate follicles. These greater androstenedione concentrations in dominant follicles of the present study were related to a greater number of LH/hCG receptors in thecal cells since these two variables were correlated ($r = 0.41$, $P < 0.01$). In addition, concentrations of estradiol were correlated with androstenedione concentrations ($r = 0.64$, $P < 0.01$) and numbers of LH/hCG receptors in thecal cells ($r = 0.36$, $P < 0.05$) in dominant and large follicles. Thus, it appears that both thecal and granulosa cell steroidogenic enzyme activity are coincidentally increased in the early developing dominant follicle.

Concentrations of IGF-I did not differ between dominant and subordinate large or small follicles during the first follicular wave of an estrous cycle in cattle, an observation not previously reported. Similarly, IGF-I concentrations in follicular fluid of large preovulatory follicles with high estradiol concentrations do not differ from those in small (< 4 mm) or medium follicles (4–7.9 mm) with low estradiol concentrations (7, 8, 32–34). Also, similar to previous reports (7, 8, 21), concentrations of IGF-I in follicular fluid tended to correlate positively with follicular diameter in cattle. In other studies in which FSH (35) and somatotropin (9) treatments were applied, follicular diameter and follicular fluid IGF-I concentrations were not correlated. Whether changes in concentrations of IGF-I in follicular fluid are the result of altered local biosynthesis or changes in diffusion of IGF-I from blood (which was on average 20% greater than in follicular fluid) remains to be elucidated. We have reported the presence of IGF-I mRNA in thecal and granulosa cells of bovine follicles (5, 6) as well as IGF-I production by cultured bovine granulosa cells (5). Thus, IGF-I is likely produced by ovarian follicles in cattle, but its contribution to the IGF-I measured in follicular fluid remains uncertain.

Reported here for the first time, concentrations of IGF-II did not differ between dominant and subordinant large follicles during the first follicular wave of an estrous cycle in cattle. However, IGF-II concentrations tended to be greater

in small than in dominant or large follicles, which is in general agreement with a previous report in ewes (22) and cattle (6). As with IGF-I, whether changes in concentrations of IGF-II in follicular fluid are a result of altered local biosynthesis or changes in diffusion of IGF-I from blood (which averaged about 50% of that measured in follicular fluid) remains to be elucidated. IGF-II mRNA is present in follicular walls of ewes (22), granulosa cells of women (36), and thecal cells of rats (37), indicating that ovarian production is likely. However, further studies will be required to determine if bovine granulosa or thecal cells produce IGF-II.

The main IGFBPs identified in plasma and follicular fluid of the present study were IGFBP-3 and IGFBP-2. Binding activity of IGFBP-3 in bovine follicular fluid was unaffected by size or stage of growth of the dominant follicle, whereas IGFBP-2 binding activity was low in the early growing dominant follicle and increased after growth had slowed. The lack of differences in binding activity of IGFBP-3 between estrogen-active and estrogen-inactive follicles agrees with observations of Echternkamp *et al.* (13). The latter authors also did not find any IGFBP-3 in bovine granulosa or thecal homogenates and suggested that IGFBP-3 was sequestered from the blood or other ovarian tissue. In support of this suggestion, the percentage of follicular fluid to plasma IGFBP-3 activity averaged 81% in the present study. The 29-kDa and 22-kDa IGFBP also demonstrated lower binding activity in early growing dominant follicles compared with subordinant small and large follicles. These results are consistent with other work examining estrogen-active preovulatory follicles in cattle (13) and women (38), and large *vs.* small follicles in sheep (22) and swine (39). The 29-kDa IGFBP is assumed to be IGFBP-5, based on immunoblotting of follicular fluid (9) and pituitary samples (40) in cattle. The 22-kDa IGFBP is most likely IGFBP-4 because the molecular weight is similar to proteins identified as IGFBP-4 in humans (41) and sheep (42). Also, mRNA for the latter two IGFBPs has been identified as being almost exclusively localized in atretic follicles of murine ovaries (43, 44). In the present study, the percentage of follicular fluid to plasma binding activity of IGFBP-2, the 29-kDa IGFBP, and the 22-kDa IGFBP averaged 131%, 238% and 131%, respectively, in small follicles, suggesting that follicles may produce these IGFBPs. Based on preliminary studies, we find that bovine granulosa and thecal cells secrete small but detectable amounts of only the 22-kDa IGFBP *in vitro* (Spicer, unpublished observations). The cause of the changes in follicular fluid IGFBP activity during follicular development in cattle is unknown but may involve specific, hormonally regulated proteases that degrade specific IGFBPs (45, 46). For example, FSH induces proteolysis of IGFBP-5 produced by cultured rat granulosa cells (45). Because follicular fluid concentrations of IGF-I did not differ among the follicle categories, the lower binding activity of IGFBPs would facilitate IGF-I bioavailability for support of further follicular growth and follicular cell differentiation (6, 14), and for inhibition of follicular cell apoptosis (12). Alternatively, IGFBPs directly inhibit FSH-induced estradiol synthesis by rat granulosa cells (47) and thus may explain, in part, why dominant follicles had greater estradiol concentrations than subordinate follicles. In support of this suggestion, estradiol concentrations in follicular fluid were negatively correlated with activity of IGFBP-2 ($r = -0.53$, $P < 0.01$), the 29-kDa IGFBP ($r = -0.39$, $P < 0.05$) and the 22-kDa IGFBP ($r = -0.38$, $P < 0.05$) but not IGFBP-3 ($r = -0.08$, $P > 0.10$) in dominant and large follicles of the present study. Moreover, concentrations of progesterone and androstenedione were not significantly correlated with any of the IGFBPs in the present study. A recent study indicated that IGFBP-3 can partially block the inhibitory effects of hCG and IGF-I on follicular cell apoptosis in cultured rat follicles (12). Further research is required to determine whether IGFBPs can directly influence follicular steroidogenesis and follicular cell apoptosis in cattle.

Numbers of IGF-I binding sites in thecal cells did not differ between early dominant and large or small subordinate follicles but were greater in late (nongrowing) dominant and large follicles than small follicles. In contrast, numbers of IGF-I binding sites in granulosa cells did not significantly differ among follicle categories although dominant and large follicles had numerically a twofold greater ($P = 0.09$) number of IGF-I receptors than small follicles. Previously, Spicer *et al.* (18) demonstrated that granulosa cells from large ($\geq$ 8 mm) bovine follicles have a severalfold greater number of IGF-I receptors than do small ($\leq$ 5 mm) follicles after culture *in vitro*. A possible reason that early dominant follicles did not significantly differ in the number of binding sites for IGF-I in thecal and granulosa cells as compared with small follicles is that the dominant follicle had just started to become dominant and some of the other smaller follicles had not yet started the process of atresia. Alternatively, because granulosa cells used after *in vitro* culture are > 95% viable whereas granulosa cells that are freshly isolated are < 30% viable (48), the ability to detect differences in cell function between type and size of follicle would be greatly accentuated using cultured cells. In addition, specific IGF-I binding to granulosa cells was greater than that found in thecal cells. This is consistent with previous *in situ* hybridization studies where IGF-I receptor mRNA was found to be more prevalent in the membrana granulosa than theca interna of rat (49) and human antral follicles (35, 50, 51). The hormonal regulation of IGF-I receptors also differs between bovine granulosa and thecal cells (11, 18, 52). For example, epidermal growth factor decreases numbers of IGF-I receptors in thecal cells, whereas epidermal growth factor has no effect on numbers of IGF-I receptors in granulosa cells cultured from large bovine follicles (11). Thus, granulosa cells which contain a greater number of IGF-I receptors than thecal cells may be more sensitive to changes in local concentrations of bioavailable IGF-I than thecal cells.

Binding sites for LH/hCG in thecal cells were greater in growing (early) and late dominant follicles, as compared with subordinate large and small follicles. In granulosa cells, LH/hCG binding sites were greater in the dominant follicle than subordinate follicles only during the late growing phase. Wandji *et al.* (10) showed that binding of ^{125}I-hCG to thecal cells increased in early antral follicles and binding to granulosa cells increased only later in the development of antral follicles, which agrees with the low level of binding observed in growing dominant follicles in our study. In bovine follicles destined to ovulate (19, 53), thecal and granulosa cells from estrogen-active follicles demonstrated in-

creased specific binding of LH/hCG as follicles grew. Recently, Xu *et al.* (54), using *in situ* hybridization, reported that FSH receptor mRNA in granulosa cells of the dominant follicle does not change between days 2 and 10 of the first follicular wave, whereas thecal and granulosa cell LH receptor mRNA increases between days 2 and 4 of the first follicular wave in beef heifers. In nonovulatory follicles (4), binding of LH/hCG to thecal and granulosa cells was highest on day 7 of the estrous cycle, which would be during the middle of the first wave of follicular growth (55, 56), at a time of maximum growth of the dominant follicle. Ireland and Roche (19) also observed that specific binding of LH/hCG to thecal cells of estrogen-active follicles increased earlier than did binding of LH/hCG to granulosa cells, which agrees with our results in that LH/hCG binding to thecal cells increased in dominant follicles before it increased in granulosa cells. This increase in thecal LH receptors could be one key factor that allows dominance to be manifested. However, the factor(s) responsible for the increase in thecal LH/hCG receptors in early dominant follicles is (are) unknown. One likely factor that stimulates the increase in thecal LH receptors is increased bioavailable IGF-I (via decreased IGFBP activity) because IGF-I increases LH/hCG receptors in cultured bovine thecal cells (11). In support of this suggestion, IGFBP-2, the 29-kDa IGFBP, and 22-kDa IGFBP activity were all negatively correlated ($r = -0.23$ to -0.29, $P < 0.05$) with numbers of thecal LH/hCG binding sites in the present study. Regardless of the stimulus, an increase in thecal LH receptors may allow for greater thecal androgen production and subsequently greater estradiol production by granulosa cells.

The early growing dominant follicles demonstrated lower IGFBP activity along with an increase in the number of thecal LH/hCG binding sites and no change in the numbers of IGF-I receptors. Previous work in our laboratory (11) showed that the increase in steroidogenesis of bovine thecal cells (from follicles ≥ 8 mm in diameter) *in vitro* in response to IGF-I and LH is due, at least partly, to increased binding sites for LH which were induced by IGF-I. However, LH (3 to 100 ng/ml) and estradiol (10 to 1000 ng/ml) had no effect on numbers of IGF-I binding sites in bovine thecal cells (Spicer, unpublished observations; 11). In contrast, both FSH and estradiol increased numbers of IGF-I binding sites in cultured bovine granulosa cells (18). Thus, greater numbers of IGF-I binding sites in granulosa *vs.* thecal cells may be due to the increase in estradiol in follicular fluid and to the increase in blood FSH observed to occur before growth of the dominant follicle (56).

In conclusion, results of the present study support the hypothesis that the procurement of LH receptors in thecal cells and increased bioavailable IGF-I in follicular fluid may be critical to the establishment of follicular dominance, whereas IGF-I receptors may only play a permissive role. Furthermore, increased intrafollicular IGFBP activity and thus decreased bioavailable IGF-I may play a role in the early stages of regression of the dominant follicle during the first follicular wave.

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