

Insulin-like growth factor binding proteins in granulosa and thecal cells from bovine ovarian follicles at different stages of development^{1,2}

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ABSTRACT: Because IGFBP inhibit IGF-stimulated cellular proliferation and differentiation, it is hypothesized that variations among IGFBP in individual follicles might contribute to the regulation of recruitment, selection, dominance, and turnover of ovarian follicles. Sources of IGFBP in fluid of bovine follicles are not well established; thus, objectives of this study were to determine levels of IGFBP binding activities and messenger RNA (mRNA) in granulosa and theca interna cells at different stages of follicular development (small [<6 mm], medium [6 to <8 mm], and large [≥ 8 mm]) and to characterize associations of these levels measured in the cells with levels of IGFBP and steroids in follicular fluid. Thecal and granulosa cells from large healthy follicles contained two- to twentyfold less ($P < 0.05$) IGFBP-2, -3, and -5 than cells from small, medium, and large atretic follicles. Thecal cells from small, medium, and large atretic follicles contained more ($P < 0.05$)

IGFBP-3 and -4 than granulosa cells from these follicles, whereas granulosa cells from these follicles contained more IGFBP-2 activity than thecal cells. Differences in IGF binding activity were paralleled by differences in levels of mRNA for the respective IGFBP. Developmental differences in IGFBP activity in follicular fluid were positively associated with activity in granulosa and/or thecal cells, with the exception of IGFBP-4, which was low in fluid from large healthy follicles but markedly increased (mRNA and binding activity) in granulosa cells from these follicles. It is concluded that developmental changes in follicular fluid IGFBP-2 and -5 binding activities seem to be controlled in part by alterations in synthesis of these IGFBP by granulosa and thecal cells, whereas diminished IGFBP-4 in fluid from large healthy follicles occurs concomitantly with increased levels of IGFBP-4 mRNA and activity in granulosa cells, implicating posttranslational regulation by specific proteases.

Key Words: Cattle, Follicle, Follicular Development, Granulosa Cells, Growth Factors, Theca Cells

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Introduction

Gonadotropin-stimulated proliferation and differentiation of ovarian granulosa and thecal cells are augmented by IGF. In turn, actions of IGF are regulated by composition of IGFBP in individual follicles (Adashi et al., 1992; Giudice, 1992). Studies in humans (Cataldo

and Giudice, 1992), swine (Mondschein et al., 1991; Howard and Ford, 1992), sheep (Monget et al., 1993), and cattle (Echternkamp et al., 1994; Stanko et al., 1994; de la Sota et al., 1996) have shown that follicular fluid IGFBP content decreases as follicles mature to the Graafian (ovulatory) stage, whereas increases in follicular fluid IGFBP content occur concomitantly with atresia. Since these original studies, a plethora of studies have characterized changes in follicular fluid levels of IGFBP at specific developmental periods in a wide range of species. However, mechanisms regulating IGFBP activities in follicular fluid remain to be fully elucidated. Mounting evidence indicates that proteolysis of specific IGFBP contribute to diminished binding activity in follicular fluid from humans (Chandrasekher et al., 1995; Conover et al., 1999; 2001) and domestic species (Mazerbourg et al., 2000; Rivera et al., 2001; Spicer et al., 2001). Thus, IGFBP activity in the fluid of a follicle is expected to reflect the balance between production and degradation. At present, research concerning intraovarian synthesis of IGFBP in cattle is limited to two studies that characterized IGFBP-2, -3,

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and/or -4 in follicles by *in situ* hybridization (Armstrong et al., 1998; Yuan et al., 1998). Activities of individual IGFBP in follicular cells have not been investigated. To provide information concerning the cellular source of IGFBP in bovine follicles, relative amounts of IGFBP activity and messenger RNA (**mRNA**) were determined in granulosa and thecal cells harvested from bovine follicles at different stages of development, and associations among these measurements with levels of IGFBP activity and steroid concentrations in follicular fluid were analyzed.

Materials and Methods

Animals and Sample Collection for IGFBP Activity

Samples used to evaluate IGFBP binding activity were obtained from ovaries collected from nine nonpregnant multiparous cycling beef cows slaughtered at the USDA, ARS, Meat Animal Research Center abattoir. Seven of the nine cows were slaughtered 24 to 48 h after an *i.m.* injection of 25 mg of PGF₂α (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI) to induce luteolysis, and the remaining two animals were slaughtered 2 to 5 d after a PG-induced ovulation. Animals were scheduled at these different time points to provide a range in the biochemical status and sizes of follicles that could be collected. Both ovaries from each cow were collected and placed in ice-cold Hanks' balanced salt solution (Sigma, St. Louis, MO) within 30 min after slaughter. Ovaries were transported to the laboratory. Follicles greater than 2 to 3 mm in diameter were dissected from ovaries and grouped by size: small (<6 mm), medium (6 to <8 mm), and large (≥ 8 mm). Small and medium follicles were pooled within size category for each animal. Large follicles were processed individually. Selection of these size categories was based on previous studies that indicated changes in hormonal and biochemical properties among these categories (McNatty et al., 1984; Kruij and Dieleman et al., 1985; Roberts and Echternkamp, 1994). Follicular fluid was aspirated from follicles with a syringe and hypodermic needle. Fluid was centrifuged 50 × *g* for 4 min at 4°C to recover aspirated granulosa cells. Follicles were bisected while immersed in a petri dish containing Hanks' buffered saline solution, and remaining granulosa cells were scraped from the follicle wall with a fine plastic loop. Buffer containing scraped granulosa cells was centrifuged and pelleted cells were combined with aspirated granulosa cells (Roberts and Echternkamp, 1994). Scraped follicle walls were placed in a new petri dish containing buffer, and theca interna cell layers were collected by microdissection away from the theca externa (Roberts and Skinner, 1990). Follicular fluid, granulosa cells, and theca interna layers were snap frozen in liquid nitrogen within 1 to 3 h after slaughter and stored frozen at -70°C until analyzed, as described subsequently. The purity of the cells collected by these procedures was greater than 95% based on culture stud-

ies of granulosa (Roberts and Echternkamp, 1994) and thecal (Roberts and Skinner, 1990) cells conducted previously.

Measurement of Steroids and IGFBP

Concentrations of estradiol and progesterone in follicular fluid were determined by RIA procedures described previously (Echternkamp et al., 1994). Subsequent to determining steroid concentrations in follicular fluid, large follicles were categorized as either large, healthy (ratio of estradiol to progesterone in follicular fluid > 1 and > 100 ng estradiol/mL of fluid) or large, atretic (any large follicle not classified as healthy).

A ligand blot procedure was used to determine IGFBP activity in follicular fluid and homogenates of granulosa and theca cells (Echternkamp et al., 1994). Samples of follicular fluid were evaluated on an equal-volume basis (1 μL). Granulosa and thecal cells were homogenized in 0.1% SDS, 1% cholic acid, and 0.1 mM phenylmethylsulfonyl fluoride solution and centrifuged briefly to remove insoluble material. Total protein concentration was determined for each sample (BCA protein assay; Pierce, Rockford, IL). Homogenates of granulosa and thecal cells were loaded onto gels based on an equal total protein basis (200 μg). Samples were subjected to one-dimensional SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Membranes were incubated in a solution containing ¹²⁵I-IGF-1 and subsequently placed with autoradiographic film. Intensity and area of individual IGFBP bands on autoradiographs were measured by scanning densitometry.

Identity of specific IGFBP in bovine ovarian follicular fluid and cell homogenates was determined by an immunoprecipitation procedure described previously (Funston et al., 1996). Representative samples of follicular fluid and cell homogenates were immunoprecipitated with antiserum against bovine IGFBP-2, human IGFBP-4, or human IGFBP-5 (Upstate Biotechnology, Inc., Lake Placid, NY). Precipitates were dissolved in SDS-PAGE gel loading buffer and subjected to the ligand blot procedure described in the preceding paragraph. Identity of specific IGFBP was then deduced by comparing location of bands from immunoprecipitated samples to bands in the same samples not subjected to antibody precipitation. Vendor specifications report cross-reactivity of the antisera against IGFBP-2 and -5 for other IGFBP as <1%. Cross-reactivity of antiserum against IGFBP-4 was reported as 50% for IGFBP-2, and <1% for other IGFBP. However, no cross-reactivity for IGFBP-2 has been observed with this IGFBP-4 antiserum when used for immunoprecipitation of several types of bovine tissues and fluids (Funston et al., 1996; Roberts et al., 1997; Keller et al., 1998).

Animals and Sample Preparation for Northern Blot Procedure

Ovaries were obtained from 39 nonpregnant cows slaughtered at random stages of the estrous cycle. Col-

lection of ovaries and dissection of follicles were carried out as described above. Total RNA was isolated from granulosa and thecal cells from follicles classified by size and follicular fluid steroid concentration as described above, with the exception that an insufficient amount of RNA was obtained from medium follicles to allow a thorough analysis of this size category. Methods and materials used for isolation of RNA and Northern blotting were described previously (Keller et al., 1998). Radioactive complementary RNA probes were transcribed from rat IGFBP-2, -4, and -5 complementary DNA templates provided by S. Shimaski, Scripps Research Institute, La Jolla, CA (see Girvigian [1994] for sequence description). A complementary RNA probe transcribed from a rat cyclophilin complementary DNA template (Danielson et al., 1988) was used to normalize loading and integrity of RNA samples. Use of these probes for evaluating gene expression in cattle was established previously (Keller et al., 1998).

Statistical Analysis

Data were analyzed using GLM procedures of SAS (SAS Inst., Inc., Cary, NC) using two different approaches. In the first approach, follicles were categorized by size, and large follicles were subdivided into healthy or atretic classifications using the criteria specified above. Differences in binding activity and steady-state levels of mRNA for each IGFBP in granulosa and thecal cell samples, and IGFBP activity in fluid from different categories of follicles were analyzed by an analysis of variance. Factors included in the model for analysis of binding activity and mRNA for IGFBP in cell samples were gel date as a blocking factor, follicle classification, cell type, and interaction of cell type \times follicle classification. For IGFBP activity in fluid, the model included gel date as a blocking factor and follicle classification. Data were transformed (log base 10) prior to analysis if variances among different classes were determined to be heterogeneous by Hartley's test (Ott, 1984). When significant ($P < 0.05$) effects of class variables were detected in analysis of variance procedures, least squares means for the significant effects were compared using the least significant differences procedure.

The second approach to analyzing data from this study was to use step-down regression procedures to evaluate relationships of individual IGFBP activities and steroid concentrations in follicular fluid with the corresponding IGFBP activities in granulosa and thecal cell homogenates. With this approach, separate regressions were run for follicles classified as either small to medium (<8 mm) or large (≥ 8 mm) without classification by steroid criteria, thereby allowing for analysis across the biochemical spectrum existing in the two size categories. The initial model included IGFBP activity in each cell type as main effects and the interaction among the two cell types. The interaction was removed from the model if the F -value was < 1 . Likewise, the main

Table 1. Follicular fluid steroid concentrations (mean \pm SE, ng/mL of fluid)

Follicle class	n	Estradiol	Progesterone
Small (<6 mm)	9	2.9 \pm 1.5 ^a	77 \pm 19 ^a
Medium (6 to <8 mm)	8	2.6 \pm 0.7 ^a	140 \pm 51 ^{a,b}
Large healthy (≥ 8 mm)	15	796 \pm 127 ^b	58 \pm 21 ^a
Large atretic (≥ 8 mm)	6	4.3 \pm 2.2 ^a	343 \pm 204 ^b

^{a,b}Means within a column without a common superscript differ ($P < 0.05$; $P < 0.06$ for progesterone in medium vs. large atretic follicles).

effect of either cell type was subsequently removed from the model if $F < 1$.

Step-down regression analyses were also performed on data from small and large follicles (atretic and healthy follicles combined) to provide insight into associations of steady-state levels of mRNA for each IGFBP within each cell type (fit as dependant variables) with transformed (log base 10) follicular fluid concentrations of estradiol, progesterone, and the interaction between these variables.

Results

Summary of Follicles Used to Characterize IGFBP Activity in Follicular Cells

Mean concentrations of estradiol and progesterone in follicular fluid from the four categories of follicles evaluated for IGFBP activity are summarized in Table 1. All cows had one or more large healthy follicles, with at least one follicle from each cow containing greater than 850 ng of E_2 /mL follicular fluid. Due to problems encountered in the laboratory, data were not obtained from the medium pool from one cow. Mean concentrations of estradiol and progesterone did not differ ($P > 0.6$) between pooled follicular fluid from small and medium follicles. Mean concentration of estradiol in fluid from large healthy follicles was two orders of magnitude greater ($P < 0.001$) than mean concentrations observed in the other follicle categories. Data were obtained from at least one or more large atretic follicles collected from all but two cows that had insufficient yield after homogenization. Mean concentration of progesterone in large atretic follicles was greater ($P < 0.06$) than mean concentrations observed in other follicle categories.

Characterization of IGFBP in Follicular Cells by Ligand Blot Procedure

A representative ligand blot illustrating IGFBP detected in homogenates of granulosa and thecal cells, and fluid from follicles is shown in Figure 1. Individual bands in Figure 1 were identified as IGFBP-2 (~34 kDa), -5 (29-32 kDa), and -4 (24, 26, and 28 kDa) by ligand blot evaluation of immunoprecipitates of follicular fluid and cellular homogenates (Figure 2). Identifi-

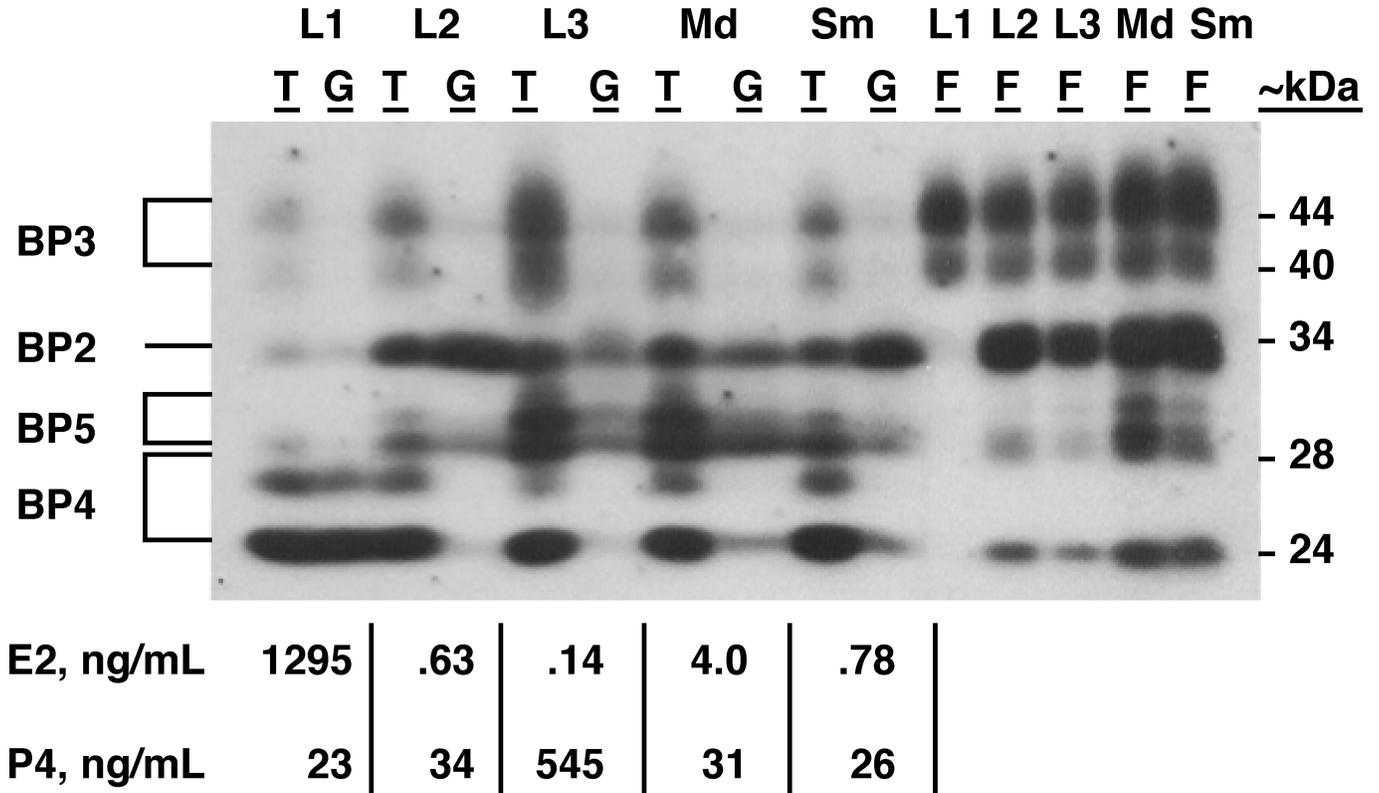


Figure 1. Representative ligand blot of IGFBP detected in follicular fluid (F), granulosa (G), and thecal (T) cells collected from individual large (≥ 8 mm in diameter; L1, L2, or L3) or pools of medium (6 to 7 mm; Md) or small (2 to 5 mm; Sm) follicles. Follicular fluid concentrations of estradiol (E2) and progesterone (P4) are shown at the bottom of the figure.

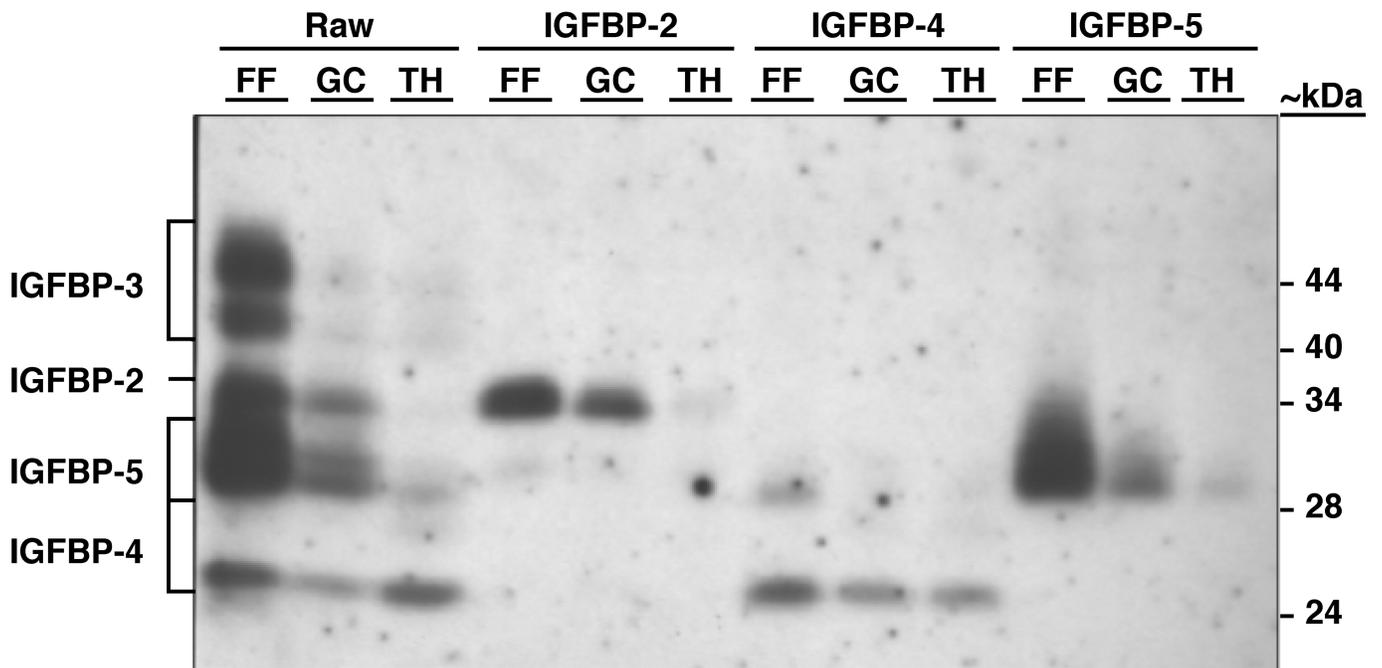


Figure 2. Ligand blot of follicular fluid (FF), granulosa cell homogenate (GC), and thecal cell homogenate (TC) from a pool of small follicles. Samples were subjected to no treatment (Raw) or immunoprecipitated with antiserum against IGFBP-2, -4, or -5 (as indicated at top of figure) before loading on gel.

cation of IGFBP-3 bands (40 and 44 kDa) is based on previous research (Funston et al., 1996).

Relative amounts of IGF binding activity (expressed as arbitrary densitometer units) detected in cellular homogenates and follicular fluid from the different follicle categories are summarized in Figure 3. Binding activity by IGFBP-3 was greater ($P < 0.04$) in thecal cells than in granulosa cells. Binding activity by IGFBP-3 was also influenced by follicle category, in which cells from large healthy follicles contained less IGFBP-3 activity than cells from small ($P < 0.05$), medium ($P < 0.12$), or large atretic ($P < 0.03$) follicle categories. For IGFBP-2, an interaction of cell type and follicle category was observed ($P < 0.02$). Granulosa cells from pools of small and medium follicles or large atretic follicles contained the greatest ($P < 0.01$) amount of IGFBP-2 activity, approximately twofold greater than that detected in the thecal cells from these follicles. In contrast, IGFBP-2 activity did not differ between granulosa and thecal cells from large healthy follicles, and cells from these follicles contained less ($P < 0.05$) IGFBP-2 activity than cells from other follicles. Binding of IGF by 32-kDa IGFBP-5 was similar ($P > 0.2$) between granulosa cells and thecal cells, but differed ($P < 0.001$) due to follicle category, with little or no activity detected in granulosa or thecal cells from large healthy follicles (not detected in 80 or 93% of samples, respectively). Distinct separation of the larger form (28 kDa) of IGFBP-4 from the smaller form of IGFBP-5 was not always possible; therefore, these proteins were grouped into one category for analysis of binding activity. Mean binding activity by 28- to 30-kDa IGFBP (IGFBP-5 and/or -4) followed a similar pattern across follicle categories as 32-kDa IGFBP-5, being lower ($P < 0.05$) in large healthy follicles, compared with other categories. Relative amounts of IGF binding by 26- and 24-kDa forms of IGFBP-4 were influenced ($P < 0.05$) by an interaction of cell type and follicle category. Thecal cells from all follicle classifications and granulosa cells from large healthy follicles contained 26- and 24-kDa IGFBP-4 binding activities much greater than granulosa cells from other follicle categories.

Statistical comparisons between IGFBP observed in cellular homogenates and follicular fluid were not made because samples were evaluated on a protein (200 μ g) or volume (1 μ L) basis, respectively. However, obvious differences were apparent between homogenates and fluid samples (see Figures 1 and 3). In follicular fluid, IGFBP-3 accounted for a much greater proportion of the total IGFBP activity than was observed in homogenates, and effect of follicle classification was not significant. In addition, the 26-kDa form of IGFBP-4 was detected in cell homogenates but was not detected in fluid from any follicles evaluated. Binding by 24-kDa IGFBP-4 was least evident in fluid from large healthy follicles, whereas the activity of this IGFBP in granulosa cells was greatest for large healthy follicles. For IGFBP-2 and 28- to 30-kDa IGFBP, binding activity in follicular fluid was less ($P < 0.05$) for large healthy follicles than

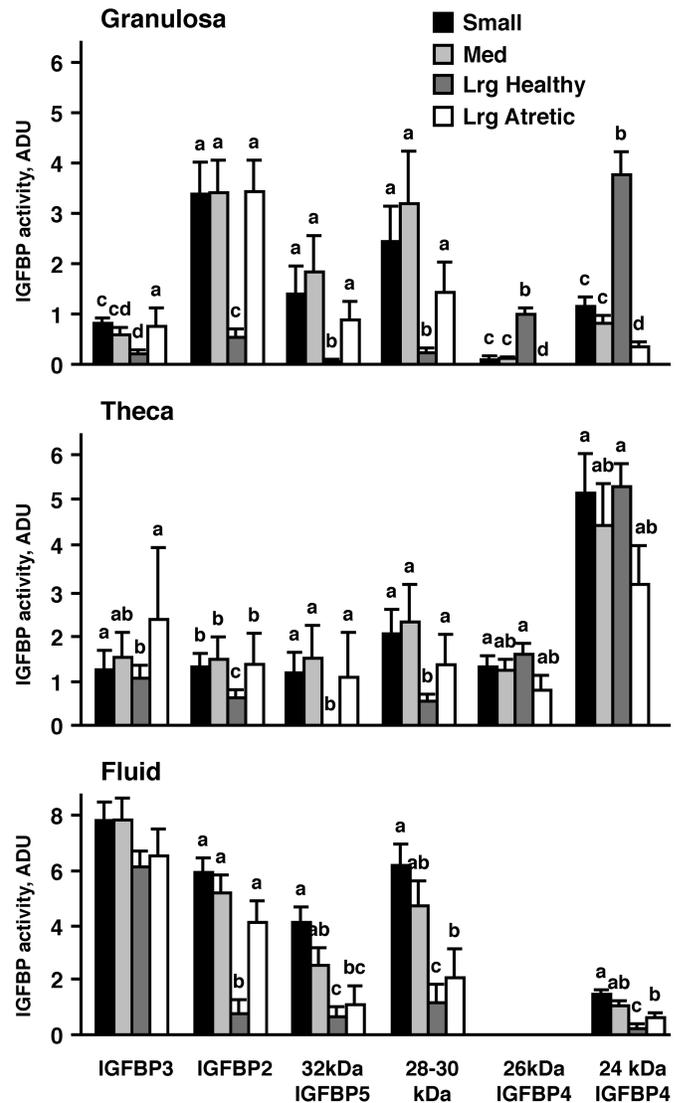


Figure 3. Relative abundance of IGFBP in granulosa (top panel) and thecal cell (middle panel) homogenates, and follicular fluid (bottom panel). Bars represent mean + SE of band intensities (arbitrary densitometer units [ADU] per unit total cellular protein or μ L fluid) from within animal pools of small ($n = 9$) and medium ($n = 8$) follicles and individual large healthy ($n = 15$) or large atretic ($n = 6$) follicles collected from nine animals. Large follicles were classified as healthy if fluid concentrations of estradiol were ≥ 100 ng/mL and concentrations of estradiol were greater than progesterone, or classified as atretic if not healthy. Bars within each category of IGFBP without a common letter differ ($P < 0.05$) from each other when data were transformed (log base 10) and compared across cell types and follicle classifications (i.e., top and middle panel) or when compared in fluid (bottom panel) from different follicle classifications. Binding in the 31- to 32-kDa area was not detected in granulosa or theca of large healthy follicles.

Table 2. The coefficient of determination (R^2) and regression coefficients obtained from regressions of IGFBP in follicular fluid (Y) on IGFBP in granulosa (GC), and thecal (TH) cells from small-to-medium ($n = 17$) or large follicles ($n = 21$) from nine cows

Y	$P <$	R^2	Mean	Intercept		+	GC		+	TH		+	GC \times TH	
				Coefficient	$P <$		Coefficient	$P <$		Coefficient	$P <$		Coefficient	$P <$
Small-to-medium follicles														
IGFBP-3	0.19	0.29	7.7	7.97	0.01		-3.3	0.4		-2.4	0.22		6.6	0.08
IGFBP-2	0.04	0.39	5.64	5.07	0.003		-0.5	0.16		1.6	0.02		—	—
32 kD BP-5	0.002	0.52	3.1	1.23	0.1		1.18	0.002		—	—		—	—
28 to 30 kD	0.001	0.68	5.1	1	0.3		1.47	0.001		—	—		—	—
24 kD	0.006	0.4	1.29	0.41	0.2		0.9	0.006		—	—		—	—
Large follicles														
IGFBP-3	0.04	0.24	5.73	5.3	0.001					0.3	0.04			
IGFBP-2	0.001	0.94	1.64	-0.16	0.5		0.66	0.001		0.63	0.03		0.22	0.03
32 kD BP-5	0.001	0.95	0.31	0.04	0.3		1.1	0.001		-0.2	0.001		—	—
28-30 kD	0.001	0.91	0.59	-0.06	0.5		1.12	0.001		—	—		—	—
24 kD	0.001	0.54	0.27	0.59	0.001		-0.12	0.001		—	—		—	—

for other follicle categories. Binding by 32-kDa IGFBP-5 followed a similar trend, except that large atretic follicles contained less activity than small follicles, but activity was not different between fluid from medium and large healthy follicles.

Regression of IGFBP in Follicular Fluid on IGFBP Activity in Cells

Results from step-down regression analyses of individual IGFBP detected in follicular fluid on the respective IGFBP detected in granulosa and thecal cells from small to medium follicles or large follicles are summarized in Table 2. In large follicles, thecal cell IGFBP-3 activity accounted for about 24% (R^2) of the variation in follicular fluid IGFBP-3 ($P < 0.04$). Levels of IGFBP-2 in fluid from small to medium follicles were associated with thecal cell IGFBP-2 activity. Levels of IGFBP-2 in fluid from large follicles were associated with changes in IGFBP-2 in both granulosa and thecal cells and the interaction between the two cell types. In small to medium follicles, 32-kDa IGFBP activity in fluid was associated positively with 32-kDa IGFBP-5 binding activity in granulosa. In large follicles, fluid 32-kDa IGFBP-5 binding activity was associated positively with 32-kDa IGFBP-5 in granulosa cells and negatively with 32-kDa IGFBP-5 in thecal cells. Activities of 28- to 30-kDa IGFBP and 24-kDa IGFBP-4 in follicular fluid were associated with the respective IGFBP in granulosa cells from both sizes of follicles. These associations were positive for each IGFBP except that 24-kDa IGFBP-4 in fluid from large follicles was negatively associated with granulosa levels of this IGFBP.

Regression of Steroids in Follicular Fluid on IGFBP Activity in Cells

Step-down regression analyses of follicular fluid concentrations of estradiol and progesterone on IGFBP activity in granulosa and thecal cells were conducted on

follicles classified as either small to medium or large to provide insight into associations among local IGFBP production and steroidogenesis (summarized in Table 3). Concentrations of estradiol in fluid from small to medium follicles were associated positively with 24-kDa IGFBP-4 in granulosa cells and tended ($P < 0.08$) to be associated positively with 32-kDa IGFBP-5 in thecal cells. Concentrations of progesterone in small to medium follicles were associated positively with the 26-kDa and 28- to 30-kDa IGFBP in granulosa cells and negatively with these IGFBP in thecal cells. Concentrations of progesterone in small to medium follicles were also associated positively with 24-kDa IGFBP-4 levels in thecal cells. Concentrations of estradiol in fluid from large follicles were associated negatively with IGFBP-3 in thecal cells, influenced by the interaction among IGFBP-2 and 28- to 30-kDa IGFBP in both granulosa and thecal cells, and were associated negatively with 32-kDa IGFBP-5 in granulosa cells. Associations among concentrations of estradiol in large follicles with either 26- or 24-kDa IGFBP-4 in granulosa and thecal cells were positive and negative, respectively. Concentrations of progesterone in fluid from large follicles were positively associated with granulosa IGFBP-5 (32 kDa), influenced by the interaction among 28- to 30-kDa IGFBP and 26-kDa IGFBP-4 in both cell types, and were negatively associated with 24-kDa IGFBP-4 in thecal cells.

IGFBP-2 mRNA

Representative Northern blots of granulosa and theca cell RNA from the different categories of follicles are shown in Figure 4. Steady-state levels of IGFBP-2 mRNA (arbitrary units/cyclophilin mRNA arbitrary units) were influenced ($P < 0.06$) by an interaction of cell type and follicular status (Figure 5). In small follicles, steady-state levels of IGFBP-2 mRNA were approximately 2.5-fold greater ($P < 0.01$) in granulosa cells than thecal cells. Levels of IGFBP-2 mRNA in both cell

Table 3. The R^2 and regression coefficients obtained from regression of follicular fluid estradiol and progesterone on individual IGFBP in granulosa (GC) and thecal (TH) cells from small to medium ($n = 17$) or large follicles ($n = 21$) from nine cows

Y Fluid steroid	X	P <	R^2	Intercept		+	GC		+	TH		+	GC × TH	
				Coefficient	P <		Coefficient	P <		Coefficient	P <		Coefficient	P <
Estradiol in small-to-medium follicles														
	32 kD BP-5	0.08	0.19	1.54	0.14	—	—	0.864	0.078	—	—	—	—	
	28 to 30 kD	0.12	0.15	1.223	0.3	—	—	0.688	0.121	—	—	—	—	
	24 kD BP-4	0.01	0.52	-1.558	0.2	4.38	0.01	—	—	—	—	—	—	
Progesterone in small to medium follicles														
	IGFBP-3	0.01	0.46	-10	0.8	170	0.01	—	—	—	—	—	—	
	IGFBP-2	0.19	0.11	152	0.01	—	—	-32.3	0.19	—	—	—	—	
	28-30 kD	0.04	0.36	84	0.05	28.7	0.02	-26.4	0.08	—	—	—	—	
	26 kD BP-4	0.02	0.43	21.5	0.07	499	0.06	-47	0.18	—	—	—	—	
	24 kD BP-4	0.07	0.21	201	0.01	—	—	19.7	0.07	—	—	—	—	
Estradiol in large follicles														
	IGFBP-3	0.06	0.2	776	0.01	—	—	-119	0.06	—	—	—	—	
	IGFBP-2	0.01	0.67	1,189	0.01	-257	0.01	-473	0.01	106	0.09	—	—	
	32 kD BP-5	0.06	0.18	657	0.01	-383	0.06	—	—	—	—	—	—	
	28 to 30 kD	0.02	0.47	1,178	0.01	-382	0.04	-641	0.04	308	0.2	—	—	
	26 kD BP-4	0.06	0.31	544	0.03	550	0.02	-260	0.14	—	—	—	—	
	24 kD BP-4	0.16	0.2	552	0.08	155	0.07	-0.87	0.27	—	—	—	—	
Progesterone in large follicles														
	32 kD BP-5	0.01	0.72	24	0.5	413	0.01	—	—	—	—	—	—	
	28 to 30 kD	0.01	0.6	192	0.08	1-30	0.7	-289	0.05	332	0.01	—	—	
	26 kD BP-4	0.04	0.42	646	0.01	-0.12-475	0.05	-372	0.03	278	0.06	—	—	
	24 kD BP-4	0.06	0.2	445	0.02	—	—	-62	0.06	—	—	—	—	

types from large atretic follicles were similar to those observed in granulosa cells from small follicles, but were greater ($P < 0.05$) than either cell type from large healthy follicles. In the regression analysis for large follicles (healthy and atretic combined), IGFBP-2 mRNA in granulosa cells was associated with follicular fluid concentrations of progesterone (Table 4). In thecal cells from large follicles, IGFBP-2 mRNA was negatively associated with follicular fluid concentrations of progesterone and estradiol (Table 4). In small follicles, changes in steady-state levels of IGFBP-2 mRNA in each cell type were affected by an interaction of both steroid concentrations in the follicular fluid (Table 4).

IGFBP-4 mRNA

Steady-state levels of IGFBP-4 mRNA were influenced ($P < 0.02$) by an interaction of cell type and follicle classification (Figure 5). Levels of IGFBP-4 mRNA were about 10-fold ($P < 0.01$) greater in thecal cells from all three follicle classifications than granulosa cells from small and large atretic follicles. However, IGFBP-4 mRNA in granulosa cells from large healthy follicles was markedly increased over that detected in granulosa cells from small and large atretic follicles, and was not different from that detected in thecal cells. Regression analysis of IGFBP-4 mRNA in granulosa cells from large follicles (atretic and healthy combined) indicated a positive association with follicular fluid estradiol concentration (Table 4). In small follicles and thecal cells from large follicles, significant associations were not

detected among steady-state levels of IGFBP-4 mRNA and follicular fluid steroid concentration.

IGFBP-5 mRNA

Two transcripts, approximately 6 and 1.8 kb in size, were detected for IGFBP-5 (Figure 4). The proportion of samples in which these transcripts were detected was greater ($P < 0.01$) for small follicle granulosa cells (27/29 and 24/29 for 6 and 1.8 kb size, respectively) than for large follicle granulosa cells (11/20 and 7/20) or thecal cells (21/58 and 8/58 for 6 and 1.8 kb transcripts in all thecal cell samples; $P > 0.9$ for thecal cells from large vs. small follicles). Steady-state level of IGFBP-5 mRNA in granulosa cells from small and large (healthy and atretic combined) follicles and thecal cells from large follicles were associated with follicular fluid steroid concentrations (Table 4). For granulosa cells from large follicles, levels of 1.8-kb IGFBP-5 mRNA were associated with follicular fluid concentrations of estradiol and progesterone, and levels of 6-kb transcript were influenced by the interaction between the two steroids. In thecal cells from large follicles, levels of the 1.8-kb transcript were associated positively with follicular fluid concentrations of progesterone. In small follicles, levels of both transcripts in granulosa cells were associated negatively with fluid concentrations of estradiol.

Discussion

Research to date indicates that IGFBP inhibit IGF actions on follicular cells (Ui et al., 1989; Spicer et al.,

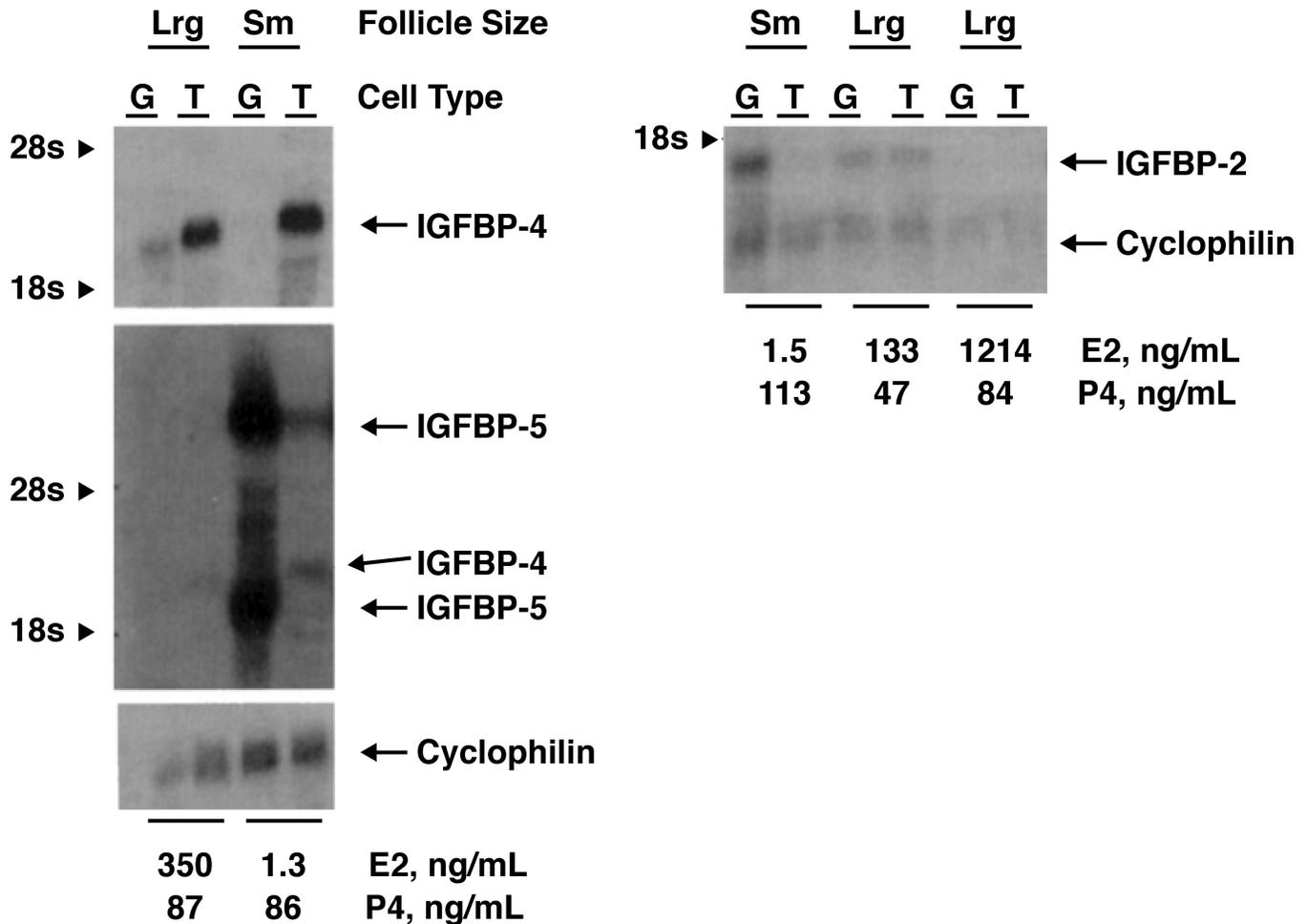


Figure 4. Representative Northern blots of granulosa (G) and thecal (T) cell RNA from small (Sm; <6 mm diameter) and large (Lrg; ≥8mm in diameter) follicles. Concentrations of estradiol (E2) and progesterone (P4) in fluid from each sample are depicted below the blots. Arrowheads at left of blots indicate location of 28s and 18s ribosomal messenger RNA as a point of reference for transcript size of IGFBP. Location of bands detected by specific complementary RNA probes for IGFBP-4, -5, and -2 are indicated on the right of the respective blots. Note that the blot shown for IGFBP-5 was probed initially for IGFBP-4, and residual bands from IGFBP-4 are evident. A probe for cyclophilin was used to adjust for unequal loading of samples.

1997; Spicer and Chamberlain 1999); thus, it is hypothesized that decreases in intrafollicular concentrations of IGFBP allow for IGF-augmented stimulation of follicular development, whereas increases or continued exposure to high concentrations of IGFBP may result in atresia or regression of follicles (Spicer and Echternkamp, 1995). As summarized in Figure 6, relative amounts of mRNA and binding activity of the different IGFBP varied by cell type and follicle size. Likewise, associations among IGFBP in the cells with IGFBP activity and steroid concentration in the follicular fluid also varied by developmental status. This novel information concerning associations among relative amounts of IGFBP in follicular fluid with the respective IGFBP in granulosa and/or thecal cells at different stages of follicular development provides insight into the potential importance of IGFBP production by indi-

vidual cell types in regulating follicular fluid levels of IGFBP.

In the present study, binding activity of IGFBP-2 was greater in granulosa than thecal cells, with the exception that levels were relatively low and indifferent in both cell types from large healthy follicles. Results from Northern blotting of IGFBP-2 mRNA concur with these results. Likewise, previous studies utilizing *in situ* hybridization also found that levels of IGFBP-2 mRNA were generally greater in granulosa than thecal cells from ovine (Besnard et al., 1996a) and bovine (Armstrong et al., 1998; Yuan et al., 1998) follicles. Although IGFBP-2 mRNA and activity were greater in granulosa cells than thecal cells, changes in follicular fluid IGFBP-2 binding activity were associated with changes in IGFBP-2 activity in thecal cells rather than granulosa cells in small follicles or both cell types in

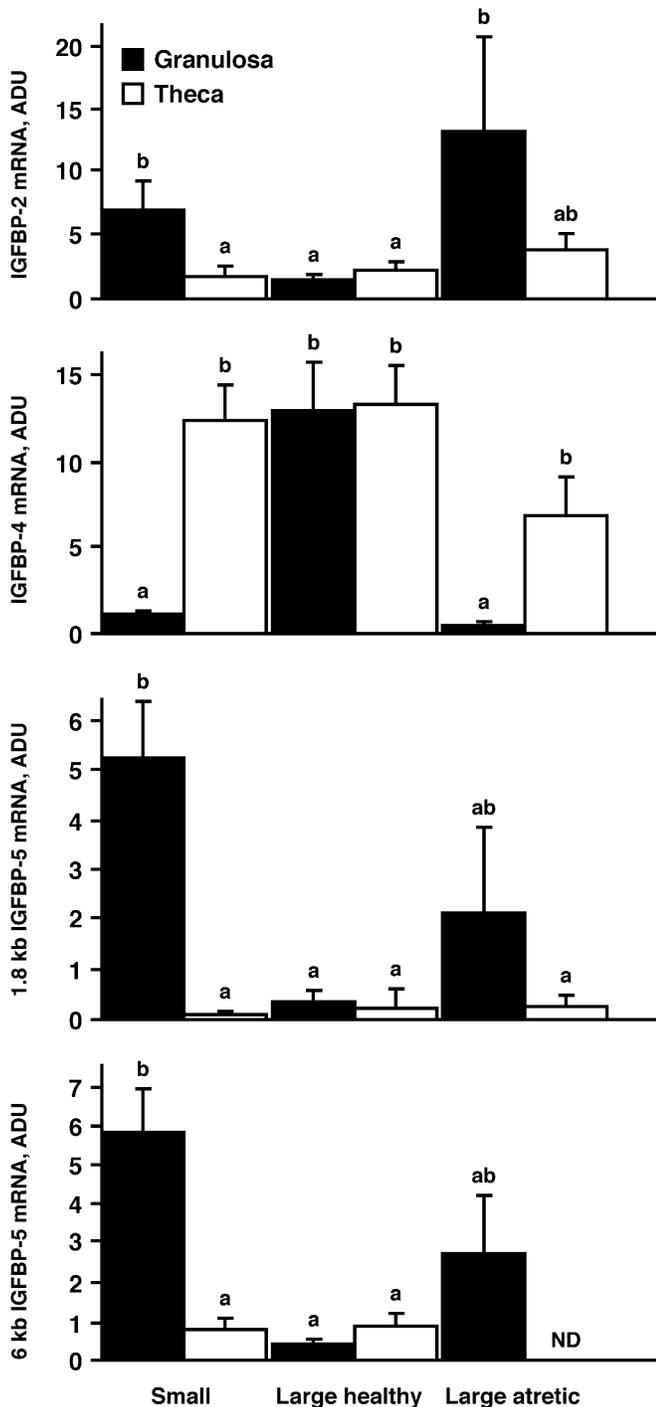


Figure 5. Mean (+ SE) levels of messenger RNA (mRNA) (arbitrary units/cyclophilin mRNA arbitrary units) detected in granulosa and thecal cells from small (<6 mm diameter), large (≥ 8 mm in diameter) healthy (>100 ng estradiol/mL fluid) and large atretic (<100 ng estradiol/mL fluid) follicles. Within each panel, bars without common letters differ ($P < 0.05$) from each other.

large follicles (see top panel Figure 6). Furthermore, variations in follicular fluid concentrations of estradiol were associated with variations in IGFBP-2 binding activity in both thecal and granulosa cells (see Table

3). Evaluating the potential regulatory pathway in the inverse fashion indicated that levels of IGFBP-2 mRNA in both thecal and granulosa cells fluctuated in association with follicular fluid levels of steroids. However, fluctuations in follicular fluid steroid concentrations accounted for a greater proportion of variation (i.e., higher R^2) for IGFBP-2 in thecal cells than in granulosa cells (shown in Table 4). These results indicate that whereas levels of transcription and activity of IGFBP-2 were generally lower in thecal cells relative to granulosa cells, changes in follicular fluid IGFBP-2 activity and steroid concentrations that occur during follicular development appear to be coupled to IGFBP-2 in thecal cells as well as in granulosa cells. Collectively, these observations indicate that intrafollicular production of IGFBP-2 by both granulosa and thecal cells may be important in regulating IGFBP-2 activity in fluid of follicles. The importance that each cell type has in regulating fluid levels of IGFBP-2 binding activity may be dependent on developmental status (i.e., size).

In previous studies on porcine (Samaras et al., 1993), ovine (Besnard et al., 1996a), and bovine (Armstrong et al., 1998; Yuan et al., 1988) follicles, increases in IGFBP-2 mRNA in granulosa and/or thecal cells were associated with advancement of morphological indicators of atresia, whereas follicular growth and increases in estradiol concentrations were associated with decreases in IGFBP-2 mRNA. Likewise, a large amount of data indicates that IGFBP-2 activity in follicular fluid is inversely associated with estradiol concentration in bovine follicles collected at specific times after prostaglandin-induced luteolysis (Echtenkamp et al., 1994; Stanko et al., 1994) or throughout the first postovulatory follicular wave (de la Sota et al., 1996; Stewart et al., 1996; Austin et al., 2001; Beg et al., 2001). The present study expands on these previous studies by providing new knowledge concerning the associations among IGFBP-2 mRNA and binding activity in granulosa and thecal cells with IGFBP-2 activity and steroid concentrations in follicular fluid, thereby contributing to the understanding of biochemical processes regulating follicular development. Collectively, these studies indicate that production of IGFBP-2 either declines with increased aromatase activity acquired during follicular development and/or increases in production of IGFBP-2 coincide with loss of aromatase activity and increases in progesterone that occur during atresia. Results from the present study provide evidence that both of these processes may occur depending on stage of development (follicle size) and cell type. Thus, a decline and subsequent increase in IGFBP-2 production coincides with establishment and loss of follicular dominance, respectively.

Although IGFBP-3 activity was detected in some granulosa and thecal cell samples, the proportion of total IGFBP activity accounted for by IGFBP-3 in follicular fluid was substantially greater than the proportion of total activity accounted for by IGFBP-3 in either cell type. Therefore, some IGFBP-3 activity in follicular

Table 4. Summary of step down regression analyses of IGFBP mRNA in granulosa or thecal cells on follicular fluid concentrations of estradiol (E₂) and progesterone (P₄) in small or large bovine follicles^{a,b}

Follicle Size	Cell Type	n	Y =	P	R ²	Mean	Intercept		Log of E ₂		Log of P ₄		Log E ₂ * Log P ₄	
							Coefficient	P	Coefficient	P	Coefficient	P	Coefficient	P
Large	Granulosa	12	IGFBP-2	0.06	0.32	4.8	-23.5	0.1			6.72	0.06	—	—
Large	Theca	16	IGFBP-2	0.02	0.49	2.4	10.5	0.001	-0.6	0.02	-1.47	0.02	—	—
Small	Granulosa	16	IGFBP-2	0.13	0.36	5.5	4.17	0.6	9.77	0.05	0.06	0.9	-1.99	0.1
Small	Theca	15	IGFBP-2	0.01	0.7	1.5	-6.4	0.01	3.41	0.03	1.73	0.002	-0.9	0.02
Large	Granulosa	21	IGFBP-4	0.02	0.24	10	-3.2	0.6	6.21	0.03	—	—	—	—
Large	Theca	27	IGFBP-4	NS ^c	—	—	—	—	—	—	—	—	—	—
Small	Granulosa	22	IGFBP-4	NS ^c	—	—	—	—	—	—	—	—	—	—
Small	Theca	32	IGFBP-4	NS ^c	—	—	—	—	—	—	—	—	—	—
Large	Granulosa	20	1.8 kb BP-5	0.04	0.32	0.5	-0.26	0.05	0.13	0.15	0.56	0.02	—	—
Large	Theca	25	1.8 kb BP-5	0.05	0.16	0.3	-0.84	0.13			0.27	0.05	—	—
Small	Granulosa	22	1.8 kb BP-5	0.08	0.15	4.7	5.29	0.001	-1.7	0.08	—	—	—	—
Small	Theca	24	1.8 kb BP-5	NS ^c	—	—	—	—	—	—	—	—	—	—
Large	Granulosa	20	6 kb BP-5	0.01	0.5	0.7	-5.31	0.09	0.98	0.11	1.35	0.03	-0.22	0.06
Large	Theca	25	6 kb BP-5	NS ^c	—	—	—	—	—	—	—	—	—	—
Small	Granulosa	22	6 kb BP-5	0.06	0.17	5.3	5.99	0.001	-1.8	0.06	—	—	—	—
Small	Theca	24	6 kb BP-5	NS ^c	—	—	—	—	—	—	—	—	—	—

^aData from medium follicles were not included in these analyses; range in estradiol was 0.06 to 13.8 ng/mL; range in progesterone was 1.3 to 959 ng/mL.

^bRange in estradiol was 0.06 to 2,352 ng/mL; range in progesterone was 9 to 400 ng/mL.

^cNS = not significant, *P* > 0.20.

fluid may originate from the circulatory system, as proposed by Grimes and colleagues (1994). Evidence for expression of IGFBP-3 within ovarian follicles is limited. Some studies reported little or no IGFBP-3 mRNA being detected in porcine (Samaras et al., 1992; 1993), bovine (Yuan et al., 1998), and human follicles (Zhou and Bondy, 1993; Voutilainen et al., 1996). In contrast, IGFBP-3 mRNA was detected in thecal cells from small follicles and in both follicular cell types from dominant human follicles (El-Roeiy et al., 1994) and vascular endothelial cells within the thecal layer of human follicles (Zhou and Bondy, 1993). In situ evaluation of bovine follicles indicated that IGFBP-3 mRNA expression exists in thecal cells from large atretic follicles and in vascular endothelial cells in theca layers (our unpublished observations), consistent with the inverse associations observed between IGFBP-3 binding activity in thecal cells and estradiol concentrations in large follicles. Further research is needed to determine if IGFBP-3 activity observed in thecal cell homogenates is derived from serum, thecal cells, and/or vascular endothelial cells within the thecal layer.

Three bands of IGFBP-4 binding activity (i.e., 24, 26, and 28 kDa) were detected in ligand blots of granulosa and thecal cells, but only two bands were detected in fluid. The 24- and 28-kDa bands represent nonglycosylated and glycosylated forms, respectively (Funston, et al., 1996). Thus, the 26-kDa form possibly represents an intermediate glycosylated form found within the cells but not in fluid. Detection of this intermediate band represents a novel finding and should be noted for future studies evaluating IGFBP in cellular or tissue homogenates. Expression of IGFBP-4 mRNA was easily detected in thecal cells from all follicles and granulosa

cells from large healthy follicles, but was not readily detectable in granulosa cells from the other follicle categories, which concurred with cellular IGFBP-4 binding activity data. Messenger RNA for IGFBP-4 was previously shown by in situ hybridization to be more abundant in thecal cells than granulosa cells of ovine (Bernard et al., 1996a) and bovine (Armstrong et al., 1998) follicles. However, these studies did not report an increase in levels of IGFBP-4 mRNA in granulosa from large healthy follicles, as observed in the present study. Detection of increased levels of mRNA and binding activity of IGFBP-4 in granulosa cells from large healthy follicles are novel observations. Although mRNA abundance and protein binding activity of IGFBP-4 were greater in theca cells than granulosa cells from small and medium follicles, changes in IGFBP-4 binding in follicular fluid were associated positively with changes in binding by this protein in granulosa cells, but not thecal cells (Figure 6). In contrast, IGFBP-4 binding activity in fluid from large follicles was associated negatively with IGFBP-4 activity in granulosa cells (Table 2 and Figure 6). Furthermore, IGFBP-4 binding activity in granulosa cells from large follicles was associated positively with follicular fluid concentration of estradiol. Thus, advances in developmental status of large follicles, as characterized by increases in follicular fluid concentrations of estradiol, appear to be accompanied by large increases in granulosa cell production of IGFBP-4 and concomitant decreases in IGFBP-4 activity in the fluid. Decreased IGFBP-4 activity in fluid from large healthy follicles from humans (Chandrasekher et al., 1995; Conover et al., 1999; 2001) and domestic species (Mazerbourg et al., 1999, 2000; Rivera et al., 2001; Spicer et al., 2001) has recently been determined to

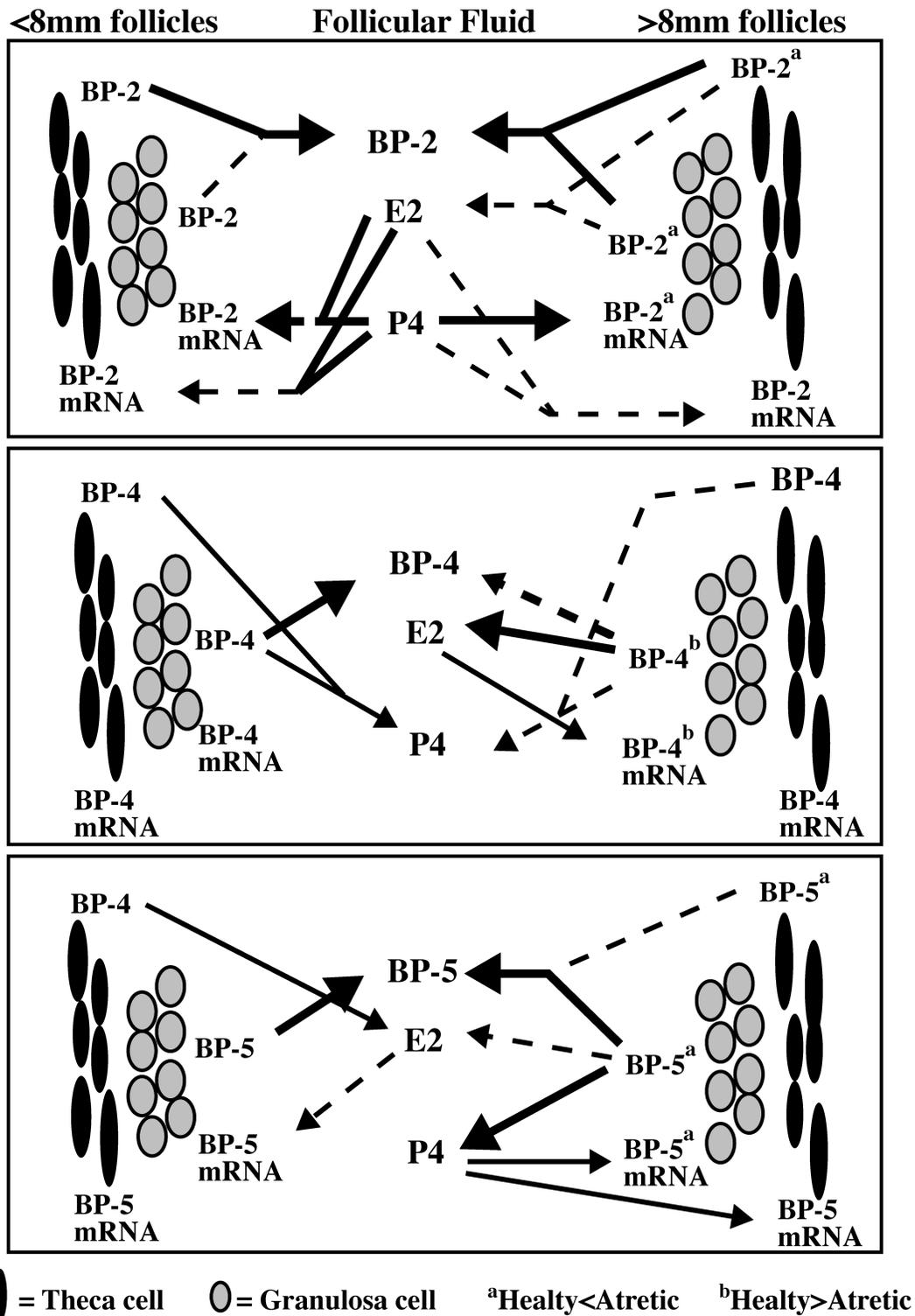


Figure 6. Summary of IGF binding activity and messenger RNA (mRNA) for IGFBP-2 (BP-2; top panel), IGFBP-4 (BP-4; middle panel), and IGFBP-5 (BP-5; bottom panel) detected in granulosa and theca cells from pools of small and medium (<8 mm; left side of panels) or individual large (>=8 mm; right side of panels) bovine follicles. Arrows depict positive (solid arrows) and negative (dashed arrows) associations determined by regression of IGFBP activity or steroid concentrations in follicular fluid (shown in center of each panel) on IGFBP activities in follicular cells, or by regression of IGFBP mRNA in each cell on steroid concentration in the fluid from follicles in each size category. Differences in text size and line thickness reflect relative differences in amounts of binding activity or mRNA and significance of associations, respectively. Relative differences in binding activity or mRNA between cells from healthy and atretic large follicles are depicted by superscripts, as defined at the bottom of figure.

result from increased proteolytic degradation of IGFBP-4. These opposing processes (i.e., increases in both synthesis and degradation) of regulating IGFBP-4 may provide an efficient method for removing a dominant follicle that has persisted beyond its useful lifespan. In this respect, large dominant follicles could be perceived as being preprogrammed to undergo atresia through a process that involves IGFBP unless otherwise interrupted by an ovulatory surge of LH.

Expression of IGFBP-5 was most abundant in granulosa cells from small and large atretic follicles and was not consistently detected in thecal cells or granulosa from large healthy follicles. This differs from results for ovine follicles, where IGFBP-5 mRNA was more abundant in the thecal cell component of all but late atretic follicles (Besnard et al., 1996a). Although very little IGFBP-5 mRNA was detected in thecal cells when compared to granulosa cells, binding activity by IGFBP-5 did not differ between thecal and granulosa cells. Potential explanations for these results include the possibility that binding activity in thecal cells is not tightly coupled to mRNA level and/or the protein may be produced elsewhere and localized within the thecal layer. Changes in binding activity by IGFBP-5 (32 kDa) in follicular fluid were associated positively with changes in granulosa levels of this protein. Taken together with results discussed previously it appears that follicular fluid levels of IGFBP-4 and -5 are associated with changes in granulosa cell activities of these binding proteins whereas fluid levels of IGFBP-2 and -3 are associated with both cell types or thecal cells, depending on developmental status.

A strong positive relationship existed between follicular fluid concentrations of progesterone and granulosa levels of IGFBP-5 in large follicles, whereas concentrations of estradiol in follicular fluid were negatively associated with IGFBP-5 in granulosa cells from large follicles (Table 3). In contrast, estradiol tended to be associated positively with levels of 32-kDa IGFBP-5 in thecal cells from small follicles. These associations are consistent with IGFBP-5 having a role in the process of atresia. In addition, IGFBP-5 may be regulated or function differently between small and large follicles. As with IGFBP-4, proteolytic activity for IGFBP-5 has been detected in follicular fluid from domestic livestock (Besnard et al., 1996b; 1997). In cattle, this activity was greater in fluid from large estrogen active follicles than subordinate large or small follicles (Spicer et al., 2001; Rivera and Fortune, 2003). Collectively, these results provide evidence that follicular fluid IGFBP-5 activity in cattle may be regulated at the level of synthesis and secretion, as well as by proteolytic degradation.

Few differences were observed among IGFBP activities within each cell type from small, medium, and large atretic follicles. Since small and medium follicles were pooled on a within animal basis, these samples likely represent a heterogeneous mixture of atretic and healthy follicles, and steroid concentrations and IGFBP activities measured for each pool would be influenced

by relative amount of tissue or fluid obtained from each individual follicle in a pool. Regression analyses performed on IGFBP and steroid concentrations in pools of small and medium follicles did identify associations among IGFBP and steroid concentrations, albeit fewer associations among these variables were observed for small and medium follicles when compared to large follicles. Therefore, pooling of small and medium follicles may not have allowed for the most robust determination of association among IGFBP in cells with IGFBP activity and steroid concentration in fluid from these samples. In contrast, large follicles were analyzed individually. Comparisons between healthy and atretic large follicles emphasize that categorizing follicles simply by size, and ignoring biochemical differences associated with variations in steroid concentration, may obscure important physiological differences. However, categorizing follicles as healthy or atretic based on qualitative criteria (a single limit in estradiol concentration and a ratio of estradiol to progesterone greater than one) assumes a threshold response and ignores the importance of variations in the biochemical processes above or below the threshold. The regression analysis overcomes this limitation and provides information concerning interrelationships among steroid concentrations and IGFBP activities in fluid with IGFBP activity or mRNA in the two cell types. This approach provides insight into which cell type or steroid (estradiol and progesterone) or interaction among the variables was most important in these relationships. The fact that both cell types or the interaction term accounted for significant sources of variation in many of the response variables analyzed indicates that simple monoculture systems may be too simplistic to study the role of the IGF system in regulating follicular development.

Implications

Changes in IGFBP activities in follicular fluid that are associated with follicular development and atresia have been well established. The present research provides evidence that binding activities of IGFBP-2 and -5 in fluid seemed to be coupled with activities of these proteins in one or both cell types. Maturation of large Graafian follicles was associated with a large increase in granulosa cell synthesis of IGFBP-4 in the face of virtually no binding activity in the fluid, supporting implications for proteolytic regulation of this IGFBP. These changes in follicular fluid IGFBP binding activities during follicular development and atresia are regulated in part by granulosa and/or thecal cells within individual follicles. The contribution that each cell type makes to IGFBP activity in fluid varied for the individual IGFBP and follicle classifications evaluated, providing further insight into complex physiological processes regulating follicular development and atresia.

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