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Estradiol and Luteinizing Hormone Regulation of Insulin-like Growth Factor Binding Protein Production by Bovine Granulosa and Thecal Cells

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To determine the effects of estradiol and luteinizing hormone (LH) on insulin-like growth factor–binding protein (IGFBP) production by bovine granulosa and thecal cells, both cell types were collected and cultured in serum-free medium with various hormone treatments, arranged in three experiments. In thecal cells, insulin stimulated ($p < 0.05$) production of IGFBP-2 and IGFBP-5, but had no effect ($p > 0.10$) on IGFBP-3 and IGFBP-4 production; LH stimulated ($p < 0.05$) production of IGFBP-2 and IGFBP-3 but had no effect ($p > 0.05$) on IGFBP-4 and IGFBP-5. Estradiol had no effect ($p > 0.10$) on IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 production by thecal cells. Production of IGFBP-2/-5 by granulosa cells from small follicles was inhibited ($p < 0.05$) by insulin, but estradiol and LH did not influence ($p > 0.10$) insulin's inhibitory effect on basal IGFBP-2/-5 production. Insulin, LH, and estradiol each inhibited IGFBP-4 production by small-follicle granulosa cells, but their effects were not additive. IGFBP-3 was not produced by small-follicle granulosa cells. In large-follicle granulosa cells, insulin and LH inhibited ($p < 0.05$) production of IGFBP-2/-5 and IGFBP-3, whereas estradiol had no effect. Insulin alone had no effect ($p > 0.10$) on production of IGFBP-4, but estradiol and LH inhibited ($p < 0.05$) production by large-follicle granulosa cells, and their effects were not additive. These results suggest that production of IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 by granulosa and thecal cells is differentially affected by hormonal stimuli.

Key Words: Insulin-like growth factor–binding proteins; estradiol; granulosa cells; thecal cells.

Introduction

During a normal estrous cycle, a network of hormonal events, which consist of peptide and steroid hormones, as well as growth factors, interact to regulate ovarian follicular growth. Through autocrine, paracrine, and endocrine mechanisms, insulin-like growth factor-1 (IGF-1) and IGF-2 stimulate growth and steroidogenesis of granulosa and thecal cells (for review see refs. *1–4*). In cattle, intrafollicular concentrations of IGF-1 remain constant during the final stages of follicular growth, whereas levels of IGF-binding proteins (IGFBPs) in follicular fluid change dramatically during folliculogenesis *(5–7)*, indicating that the amount of IGF binding to its receptor, and ultimately follicular growth, may be dictated by IGFBPs. Specifically, IGFBP-2, IGFBP-4 and IGFBP-5 are predominately localized in large subordinate and small bovine follicles, with little to no activity of these three IGFBPs detected in the follicular fluid of dominant nonovulatory and ovulatory follicles *(5–7)*. During the middle stage of the estrous cycle (approx d 10), when the dominant nonovulatory follicle is beginning to undergo atresia, levels of IGFBP-2, IGFBP-4, and IGFBP-5 increase in the dominant follicle *(7)*. The varying pattern of these IGFBPs within the follicle during the estrous cycle suggests that IGFBP-2, IGFBP-4, and IGFBP-5 are hormonally regulated. By contrast, levels of IGFBP-3 remain constant during folliculogenesis *(5–7)*.

How hormones and growth factors regulate follicular IGFBP production appears to vary among species and ovarian cell type. In cultured porcine granulosa cells, follicle-stimulating hormone (FSH) inhibits, insulin stimulates, and epidermal growth factor (EGF) has no effect on IGFBP-2 production *(8,9)*. In cultured rat granulosa cells, FSH decreases the amount of IGFBP-4 protein *(10)* and its mRNA *(11)*. However, FSH has no effect whereas insulin and EGF inhibit IGFBP-2/-5 production by cultured bovine granulosa cells *(12)*. Luteinizing hormone (LH) treatment increases IGFBP-4 production by cultured ovine thecal cells *(13)* and increases IGFBP-5 mRNA levels in cultured human thecal cells *(14)*. In bovine granulosa cells, glucocorticoids decrease IGFBP-4 and IGFBP-2/-5 production, whereas glucocorticoids have no effect on IGFBP-4 production by bovine thecal cells *(12)*. Whether estradiol alters

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IGFBP production by granulosa or thecal cells of any species is not known. IGFBPs inhibit IGF-1-induced steroidogenesis and mitogenesis of bovine granulosa and thecal cells *(15–17)*, and IGFBP levels decrease as estradiol levels increase *(7,18–20)*. Thus, hormonal changes may alter the action of IGFs on granulosa and thecal cells via changes in IGFBPs.

The present study was undertaken to compare the hormonal regulation of IGFBP production by bovine granulosa and thecal cells, with specific focus on the effects of estradiol and LH and their interaction with insulin.

Results

Experiment 1: Effect of Insulin, LH, and Estradiol on Thecal Cell IGFBP Production

Experiment 1 was designed to determine whether insulin, LH, and/or estradiol affect IGFBP production by thecal cells of large follicles. Thecal cells from large follicles (≥8 mm) were cultured for 48 h as described in Materials and Methods. For the subsequent 24 h, cells were cultured in serum-free medium containing six treatments: no additions (control), insulin (100 ng/mL), LH (100 ng/mL), estradiol (500 ng/mL), LH (100 ng/mL) plus estradiol (500 ng/mL), and insulin (100 ng/mL) plus estradiol (500 ng/mL). These doses of insulin and LH have previously been reported to be maximal for stimulating thecal steroidogenesis *(4,16,21)*. Because estradiol was dissolved in ethanol, all treatments without estradiol received the same volume of ethanol (final ethanol content = 0.003%) as a vehicle control.

Three major IGFBPs produced by thecal cells were a doublet at 42–44 kDa (IGFBP-3), a doublet at 27–29 kDa (IGFBP-5), and a doublet at 20–22 kDa (IGFBP-4); a minor band at 34 kDa (IGFBP-2) was also detected (Fig. 1A). Estradiol alone had no effect ($p > 0.05$) on IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 production by thecal cells, whereas LH alone increased ($p < 0.05$) production of IGFBP-3 (Fig. 2). Combined treatment of LH and estradiol increased ($p < 0.05$) IGFBP-2 and IGFBP-5 production. Insulin alone increased ($p < 0.05$) thecal IGFBP-2 and IGFBP-5 production (Fig. 2). Thecal cell production of IGFBP-4 was not influenced ($p > 0.10$) by any treatment (Fig. 2). Numbers of thecal cells averaged 3.99 and 2.99 ± .05 $\times 10^5$ cells per well in the presence and absence of insulin, respectively, and were not affected ($p > 0.10$) by estradiol or LH.

Experiments 2 and 3: Effect of Insulin, LH, and Estradiol on IGFBP Production by Granulosa Cells from Small and Large Follicles

Experiments 2 and 3 were designed to determine whether insulin, LH, and/or estradiol affect IGFBP production by less differentiated, small-follicle and moderately differentiated, large-follicle granulosa cells, respectively. In cattle, small-follicle granulosa cells have fewer LH receptors and a weaker response to LH than large-follicle granulosa cells

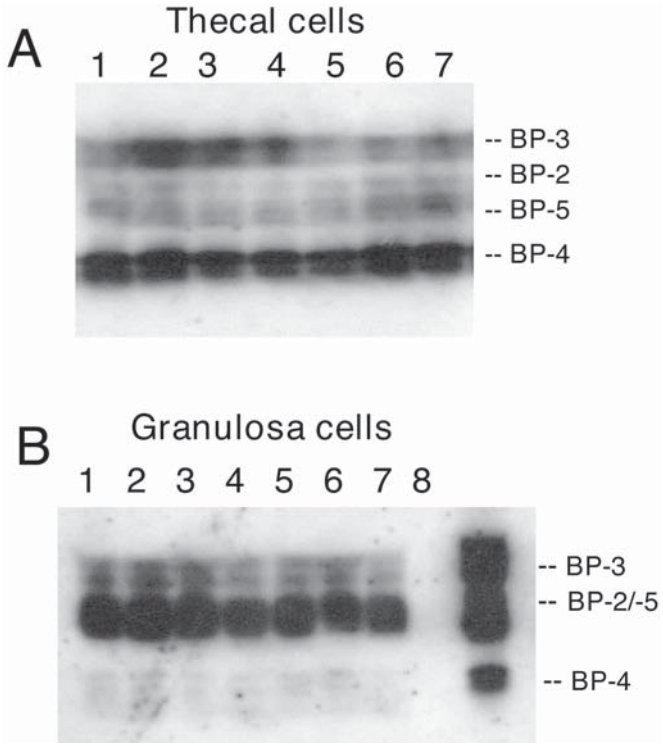

Fig. 1. (**A**) Representative ligand blot of large-follicle thecal cell IGFBP production after 14-d X-ray exposure time. Lane 1, cells treated with 500 ng/mL of estradiol; lane 2, cells treated with 100 ng/mL of LH; lanes 3 and 4, cells treated with estradiol plus LH; lanes 5 and 6, cells treated with 100 ng/mL of insulin; lane 7, cells treated with insulin plus estradiol. (**B**) Representative ligand blot of large-follicle granulosa cell IGFBP production after 14-d X-ray exposure time. Lane 1, untreated cells; lane 2, cells treated with 100 ng/mL of insulin; lane 3, cells treated with 500 ng/mL of estradiol; lanes 4 and 5, cells treated with insulin plus estradiol; lanes 6 and 7, cells treated with 100 ng/mL of LH; lane 8, blank lane; lane 9, pooled bovine follicular fluid.

(7,22). After the initial 48-h incubation period in medium containing 10% fetal calf serum (FCS), granulosa cells from either small (1–5 mm) or large (≥8 mm) follicles were incubated for another 24 h in serum-free medium containing six treatments: no additions (control), estradiol (500 ng/mL), insulin (100 ng/mL), LH (100 ng/mL), LH (100 ng/mL) plus estradiol (500 ng/mL), and insulin (100 ng/mL) plus estradiol (100 ng/mL). These doses of insulin, LH, and estradiol have previously been reported to be maximal for stimulating or inhibiting granulosa cell steroidogenesis *(4, 22–24)*. Because estradiol was dissolved in ethanol, all treatments without estradiol received the same volume of ethanol (final ethanol content = 0.003%) as a vehicle control.

The major IGFBP produced by small-follicle granulosa cells (experiment 2) was a 27- to 34-kDa IGFBP (IGFBP-2 and IGFBP-5); minor bands were detected for a 20- to 22-kDa IGFBP (IGFBP-4), and no bands were detected for IGFBP-3. Insulin alone, decreased ($p < 0.01$) IGFBP-2/-5 and IGFBP-4 production. Estradiol alone decreased ($p < 0.01$)

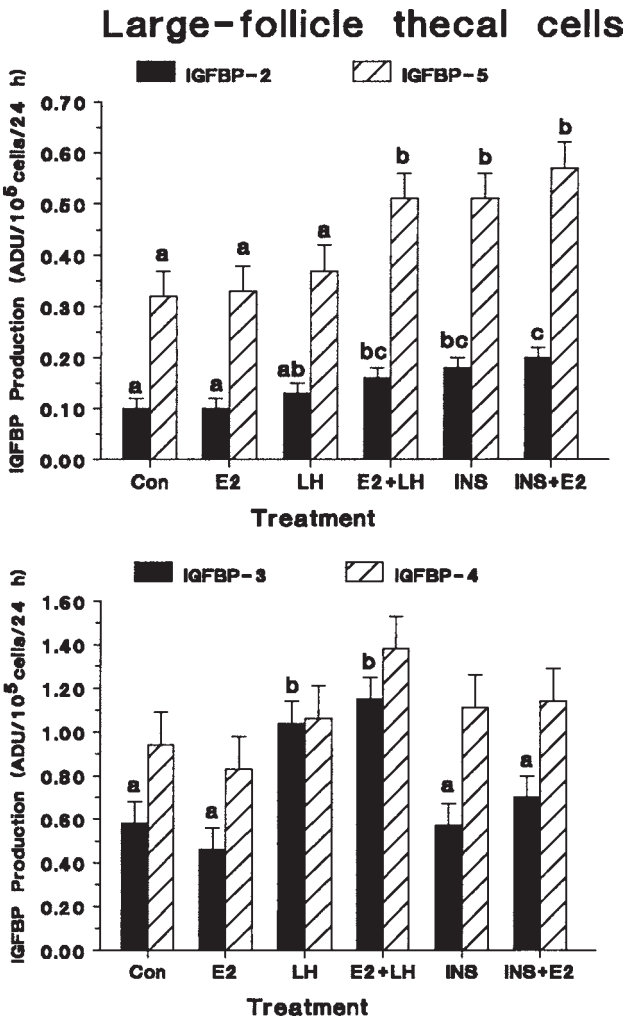

Fig. 2. Effect of insulin, LH, and estradiol on IGFBP production (expressed as arbitrary densitometric units [ADU]/[10^5 cells·24 h]) by thecal cells from large (≥8 mm) follicles (experiment 1). Thecal cells were cultured for 2 d in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin (INS), 100 ng/mL of LH, 500 ng/mL of estradiol (E2), 100 ng/mL of LH plus 500 ng/mL of E2 (E2 + LH), 500 ng/mL of estradiol plus 100 ng/mL of INS (INS + E2), or no additions (Con) for an additional 24 h. Values are means ± SEM from three separate experiments done in triplicate. Within a panel, means without a common letter differ ($p < 0.05$).

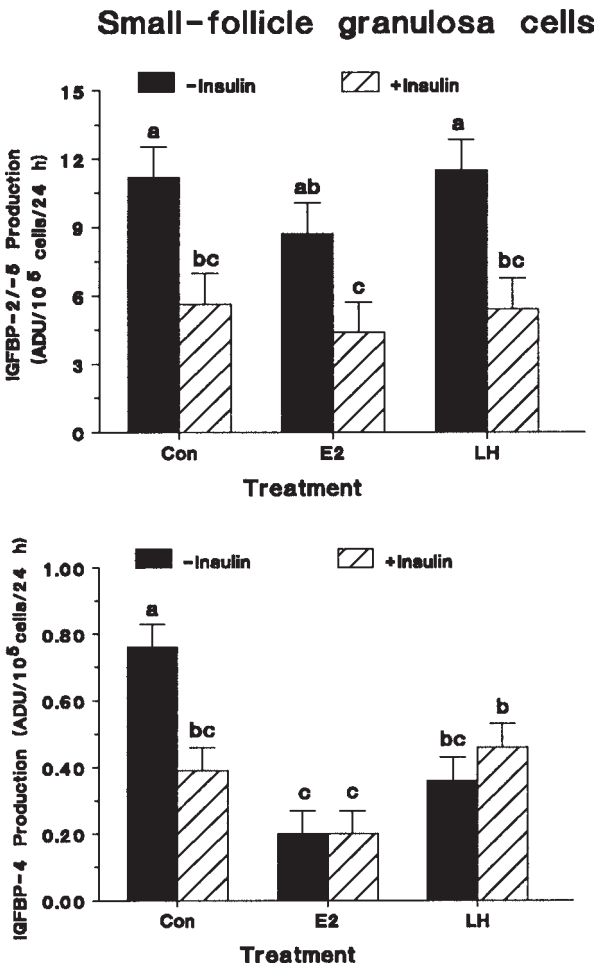

Fig. 3. Effect of insulin, LH, and estradiol on IGFBP production (expressed as ADU/[10^5 cells·24 h]) by granulosa cells from small (1–5 mm) follicles (experiment 2). Granulosa cells were cultured for 2 d in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin, 500 ng/mL of estradiol (E2), 100 ng/mL of insulin plus 500 ng/mL of estradiol, 100 ng/mL of LH, 100 ng/mL of insulin plus 100 ng/mL of LH, or no additions (Con) for an additional 24 h. Values are means ± SEM from three separate experiments done in triplicate. Within a panel, means without a common letter differ ($p < 0.05$).

IGFBP-4 production but had no effect on IGFBP-2/-5 production (Fig. 3). Treatment with LH alone decreased ($p < 0.05$) production of IGFBP-4 but had no effect ($p > 0.10$) on IGFBP-2/-5 production (Fig. 3). Neither LH nor estradiol further augmented ($p > 0.05$) the inhibitory effect of insulin on production of IGFBP-2/-5 or IGFBP-4 (Fig. 3). Numbers of granulosa cells averaged 0.96 and 0.44 ± 0.04 × 10^5 cells per well in the presence and absence of insulin ($p < 0.05$), respectively, and were not affected ($p > 0.10$) by estradiol and LH.

The major IGFBP produced by large-follicle granulosa cells (experiment 3) was IGFBP-2/-5; minor bands were detected for IGFBP-3 and IGFBP-4 (Fig. 1B). As observed for small-follicle granulosa cells, estradiol had no effect ($p > 0.05$) on basal IGFBP-2/-5 production, whereas estradiol decreased ($p < 0.05$) IGFBP-4 production by large-follicle granulosa cells (Fig. 4). LH alone inhibited ($p < 0.05$) production of IGFBP-2/-5 and IGFBP-4 (Fig. 4). The combined treatments of LH and insulin or estradiol and insulin decreased IGFBP production to levels seen with

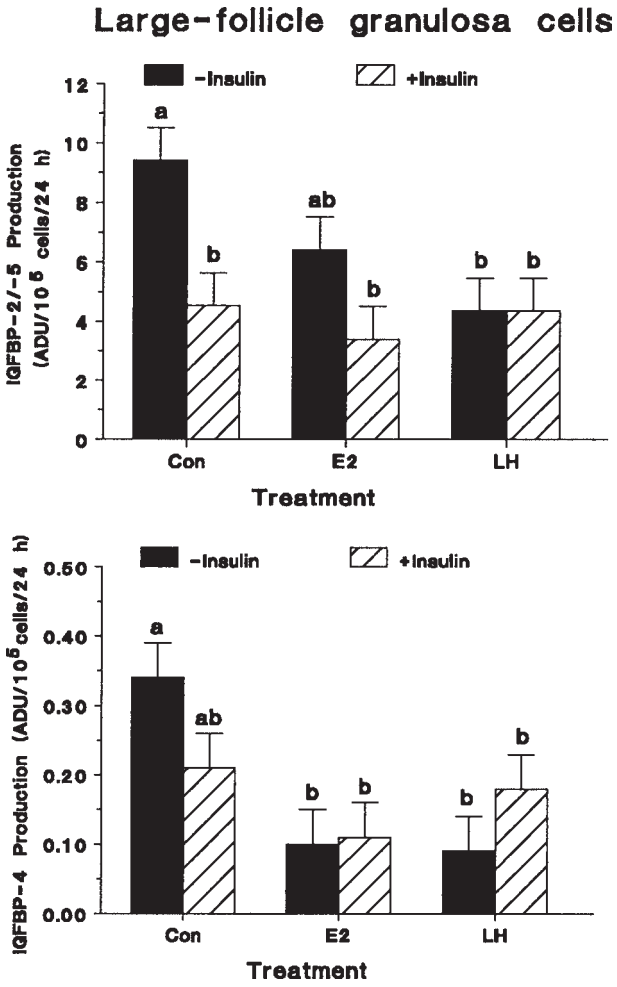

Fig. 4. Effect of insulin, LH, and estradiol on IGFBP production (expressed as ADU/[10^5 cells·24 h]) by granulosa cells from large (≥8 mm) follicles (experiment 3). Granulosa cells were cultured for 2 d in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin, 500 ng/mL of estradiol (E2), 100 ng/mL of insulin plus 500 ng/mL of estradiol, 100 ng/mL of LH, 100 ng/mL of insulin plus 100 ng/mL of LH, or no additions (Con) for an additional 24 h. Values are means ± SEM from three separate experiments done in triplicate. Within a panel, means without a common letter differ ($p < 0.05$).

singular treatments (Fig. 4). Production of IGFBP-3 averaged 1.53 ± 0.31 ADU/(10^5 cells·/24 h) and was not affected ($p > 0.05$) by estradiol, insulin, or LH (data not shown). Numbers of granulosa cells averaged 0.95 and 0.61 ± 0.05 × 10^5 cells per well in the presence and absence of insulin ($p < 0.05$), respectively, and were not affected ($p > 0.10$) by estradiol or LH.

Discussion

The results of the present study revealed the following: A 42- to 44-kDa (IGFBP-3), a 34-kDa (IGFBP-2), a 27- to 29-kDa (IGFBP-5), and a 20- to 22-kDa IGFBP (IGFBP-4) were produced by thecal cells while a 27- to 34-kDa (IGFBP-2/-5) and a 20- to 22-kDa IGFBP (IGFBP-4) were consistently produced by granulosa cells. Insulin and LH enhanced whereas estradiol did not affect production of IGFBP-2 and IGFBP-5 by thecal cells. LH had no effect on small-follicle granulosa cell IGFBP-2/-5 production but decreased large-follicle granulosa cell IGFBP-2/-5 production. LH and estradiol decreased production of IGFBP-4 by granulosa cells of both small and large follicles. The inhibitory effects of insulin, LH, and estradiol on granulosa cell IGFBP-2/-5 and IGFBP-4 production were not additive. Thecal IGFBP-3 production was increased by LH whereas granulosa IGFBP-3 production and thecal IGFBP-4 production were unresponsive to hormone treatment.

Based on immunoblotting techniques from other laboratories, the 42- to 44-kDa IGFBP is likely IGFBP-3, the 34-kDa one is likely IGFBP-2, the 27- to 29-kDa one is likely IGFBP-5, and the 20- to 22-kDa one is likely IGFBP-4 *(5, 6,25)*. Based on previous research *(11,26–28)*, the multiple bands between 20 and 22 kDa may represent different deglycosylated forms of IGFBP-4. In addition, the 27- to 34-kDa IGFBP produced by the granulosa cells is likely a composite of two IGFBPs: IGFBP-2 and IGFBP-5; because the bands identifying the two different IGFBPs were indistinguishable on the ligand blots, the IGFBPs were scanned and analyzed as one.

The IGFBPs most influenced by hormone treatments were IGFBP-2, IGFBP-3, and IGFBP-5 by thecal cells and IGFBP-2/-5 and IGFBP-4 by granulosa cells. By contrast, IGFBP-4 produced by the thecal cells and IGFBP-3 produced by granulosa cells were not influenced by any hormone regimen. The lack of response of IGFBP-4 and IGFBP-3 to hormone treatments in cultured thecal and granulosa cells, respectively, in the current study may suggest that the constitutive expression of these two IGFBPs from these two cell types do not contribute to changes in their levels in follicular fluid during the estrous cycle. In bovine follicles, *in situ* hybridization (ISH) indicates that IGFBP-2 mRNA is primarily expressed in granulosa cells (vs thecal cells), whereas IGFBP-4 mRNA is primarily expressed in thecal cells (vs granulosa cells) *(29)*. Similarly, IGFBP-2 mRNA is detected by ISH in granulosa cells but not thecal cells of bovine subordinate (and to a lesser extent, dominant) follicles *(30)*. Similar to previous results in rats *(11,31)*, bovine thecal cells, but not granulosa cells, consistently produced IGFBP-3. Using reverse transcriptase polymerase chain reaction, IGFBP-3 mRNA was detected in large-follicle thecal and granulosa cells but not small-follicle granulosa cells in cattle *(32)*. Using ISH techniques, IGFBP-3 mRNA was detected in granulosa cells of only 2 of 28 bovine follicles but was not detectable in thecal cells *(30)*. In addition, IGFBP-3 mRNA is low or undetectable in ovine follicles *(33)* and bovine ovaries *(34)*. ISH experiments in sheep follicles indicate that the IGFBP-2 mRNA is primarily expressed in granulosa cells, whereas IGFBP-4 and IGFBP-5 mRNA are expressed in thecal cells in healthy ovine follicles *(35)*.

However, both IGFBP-2 and IGFBP-5 are secreted by cultured ovine granulosa cells *(13,36)*. Rat theca, but not granulosa cells, contain IGFBP-2 mRNA whereas the converse is true for the mouse *(37–39)*. In the pig, IGFBP-2 mRNA is found in both thecal and granulosa cells *(40)*. In both the rat and mouse, IGFBP-4 mRNA is found mainly in granulosa cells with some expression in thecal cells *(31,39,41)*, whereas IGFBP-5 mRNA is found in granulosa but not thecal cells *(39,42)*. Thus, species differences, size of follicle, and variability in sensitivity of mRNA detection techniques may account for inconsistencies among studies in terms of which cell type produces a particular IGFBP in addition to the physiologic status of the follicle.

For the first time, we have found that estradiol inhibits production of IGFBP-4 by granulosa cells from both large and small follicles with no effect on IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 production by thecal cells. Similarly, IGFBP-4 production was decreased with estradiol in cultured osteoblast cells *(43,44)*. Interestingly, estradiol treatment in the absence or presence of insulin had no significant effect on IGFBP-2/-5 production by granulosa cells from either small or large follicles. Consistent with this finding, Putowski et al. *(45)* observed that diethylstilbestrol treatment in hypophysectomized rats had no effect on total ovarian IGFBP-5 mRNA. Because follicular fluid levels of IGFBP-2, IGFBP-4, and IGFBP-5 decrease concomitantly with selection of the dominant follicle in cattle *(7,19,20)*, and these IGFBP mRNA levels are differentially regulated, the decrease in IGFBP-2, IGFBP-4, and IGFBP-5 is likely regulated via different mechanisms. One additional mechanism may be via IGFBP proteolysis *(46,47)*. Specifically, proteolysis of IGFBP-4 and IGFBP-5 but not IGFBP-2 and IGFBP-3 is increased in dominant vs subordinate follicles of cattle *(46)*. Consistent with the lack of effect of estradiol on IGFBP-3 production by large-follicle granulosa and thecal cells in the present study, estradiol had no influence on IGFBP-1 production by MCF-7 cells *(48)*. Because increased IGFBP-2, IGFBP-4, and IGFBP-5 production is associated with follicular atresia *(2,3)*, the estradiol-induced decrease in IGFBP-4 production in granulosa cells may help maintain or further stimulate follicle growth. The effect of estradiol on granulosa cell IGFBP-4 production is likely mediated via genomic action since mRNA for estrogen receptor-β is present in bovine granulosa cells *(49)*. Additionally, direct inhibitory effects of estradiol on progesterone production by bovine thecal and granulosa cells in vitro have been reported *(22,50)*.

Collectively, these results imply that estradiol may be involved in stimulating growth of ovarian follicles in cattle via an inhibitory effect on IGFBP-4 production by granulosa cells. The physiologic consequences of the inhibitory effect of estradiol on granulosa cell IGFBP-4 synthesis could allow for more bioavailable IGF during selection of the early dominant follicle when estradiol levels are greatest *(7,19,20)*.

Interestingly, in the present study, LH increased IGFBP-3 production by thecal cells but not by granulosa cells; insulin and estradiol were without effect. LH or estradiol alone had no effect on IGFBP-2 production by thecal cells. Similarly, LH had no effect on IGFBP-2 production in rat *(38)*, human *(14)*, and ovine *(13)* thecal cells. By comparison, LH had no effect on IGFBP-3 protein and mRNA levels in cultured human thecal cells *(14)* and granulosa cells *(51,52)*, or on IGFBP-4 production by rat thecal cells *(38)*. However, LH treatment caused a dose- and time-dependent increase of IGFBP-4 production in cultured ovine thecal cells with 4- and 6-d treatments needed to maximize the LH effect *(13)*. In cultured bovine thecal cells, IGFBP-4 mRNA was increased after 4 d of treatment with 100 ng/mL of LH *(37)*. In addition, LH treatment increased IGFBP-5 mRNA levels in cultured human thecal cells, and human chorionic gonadotropin (hCG) treatment of human granulosa cells increased IGFBP-4 production *(14,51)*. In vivo, hCG treatment increased total ovarian IGFBP-4 mRNA in immature rats *(53)*. By contrast, LH decreased IGFBP-3 production by human granulosa cells *(54)* but did not alter IGFBP-3 production by bovine granulosa cells in the present study.

These studies suggest that the influence of LH on IGFBP production may be dependent on species, which specific IGFBP is measured, and/or culture conditions (e.g., duration of treatment). Although IGFBP-3 is the primary IGFBP found in bovine follicular fluid *(6,7,18)*, the levels of IGFBP-3 in follicular fluid reflect those found in the systemic circulation of cattle *(7,18)*, suggesting that the majority of IGFBP-3 found in follicular fluid may be transuded from blood. The lack of hormone responsiveness of IGFBP-3 production by large-follicle granulosa cells, and the lack of IGFBP-3 produced by small-follicle granulosa cells support this conclusion.

Insulin treatments in the present study increased IGFBP-2 and IGFBP-5 production by thecal cells but decreased IGFBP-2/-5 production by granulosa cells from both small and large follicles. The present results extend our previous findings *(12)* to granulosa cells of large follicles. By comparison, insulin inhibited IGFBP-4 production by granulosa cells of small follicles but had no effect on IGFBP-4 production by large follicles. Similar to what was found for large-follicle granulosa and thecal cells of the present study, insulin had no effect on production of IGFBP-3 protein by cultured human granulosa cells *(54)*. Insulin's effect on IGFBP-4 may be dose dependent because high insulin doses (≥100 ng/mL) increased IGFBP-4 production whereas low doses (≤10 ng/mL) did not affect IGFBP-4 production by human endometrial stromal cells *(55)*. In contrast to IGFBP-4, high doses of insulin decreased IGFBP-2 production and/or its mRNA by rat hepatocytes but had no effect in bone cells *(56)*. Thus, the specific effect of insulin on the production of a particular IGFBP may be dependent on the cell type and/or concentration of insulin used. Interestingly, the same dose of insulin had opposing effects on thecal and

granulosa cell IGFBP-2/-5 production in the present study. Theoretically, the latter observation would imply that the net effect of insulin on follicular IGFBP-2 and IGFBP-5 production is negligible in the cow. Alternatively, during good nutrition when insulin levels are elevated, increased thecal IGFBP-2/-5 may maintain a higher local concentration of IGF-1 and, at the same time, reduce the amount of IGFBP-2/-5 produced by granulosa cells; the net effect would be increased IGF-1 stimulated granulosa cell functions.

The current and previous results indicate that the mechanism by which intrafollicular IGFBP production is regulated is likely through a network of hormones. Because IGFBP levels decrease during follicular growth and increase during follicular atresia, changes in several reproductive and metabolic hormone concentrations during the estrous cycle could alter ovarian IGFBP production and/or protease activity. In a growing dominant follicle with increased concentrations of estradiol, the inhibitory effect of LH and estradiol on granulosa cell IGFBP-4 production would result in an increase in bioavailable IGF and, in turn, allow continued ovarian follicular growth and development. The physiologic role of insulin in regulating IGFBP-2/-5 production is less clear because of its opposing effects on thecal and granulosa cells and will require further elucidation.

Materials and Methods

Reagents and Hormones

The following reagents and hormones were used: Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, gentamicin, glutamine, sodium bicarbonate, purified bovine insulin (28 U/mg), FCS, estradiol, protease, collagenase, hyaluronidase, DNase, and acrylamide, all obtained from Sigma (St. Louis, MO); bovine LH (L1914, LH activity 2.0 × NIH-LH-S1 U/mg) obtained from Scripps (San Diego, CA); bovine serum albumin (BSA) (protease free) obtained from Intergen (Purchase, NY), which was prescreened from several lots and determined to contain undetectable amounts of insulin, IGF-1, and IGFBPs; recombinant bovine IGF-2 obtained from Monsanto (St. Louis, MO); 20% (w/v) sodium dodecyl sulfate (SDS) solution from Amresco (Solon, OH); and nitrocellulose transfer membrane (0.45-μm pore size) from Midwest Scientific (St. Louis, MO).

Cell Culture

Ovaries from pregnant and nonpregnant beef and dairy cattle were collected at a local commercial abattoir, placed in saline (0.15 *M* NaCl), and transported to the laboratory (<120 min), where small (1–5 mm) and large (≥8 mm) follicles were aspirated separately using a 20-gage needle (38.1 mm) and 3-mL syringe to collect granulosa cells as previously described *(22,57)*. Additional granulosa cells were obtained from large follicles after bisection and manual scraping of granulosa cells from the follicle wall. The aspirated and scraped granulosa cells from large follicles were combined and washed three times in serum-free medium by centrifuging at 200*g* for 5–7 min. The cells were then resuspended in medium containing 1.25 mg/mL of collagenase and 0.5 mg/mL of DNase to prevent clumping.

Thecal cells were collected from large follicles (≥8 mm) by dissection as previously described *(21,57)*. Briefly, after isolation and removal of granulosa cells, the theca interna was torn into small pieces and enzymatically digested for 1 h at 37ºC on a rocking platform. The large follicles used were those that appeared healthy (i.e., well vascularized and having transparent follicular fluid). Any tissue not digested after the incubation period was removed via filtration through a syringe filter holder with a metal screen (149-μm mesh; Gelman, Ann Arbor, MI). The thecal cells were then washed in serum-free medium by centrifuging at 50*g* for 4 min and resuspended in medium containing 1.25 mg/mL of collagenase and 0.5 mg/mL of DNase. Thecal cells from large follicles were used because they retain LH, insulin, and IGF-1 responsiveness *(4,21)*. The numbers of viable small-follicle granulosa, large-follicle granulosa, and thecal cells were determined by the trypan blue exclusion method and averaged 85.0 ± 5.6, 94.5 ± 3.2, and 90.2 ± 2.2%, respectively.

Medium consisted of a 1:1 mixture of DMEM and Ham's F-12 containing 0.12 m*M* gentamicin, 2.0 m*M* glutamine, and 38.5 m*M* sodium bicarbonate. Approximately 3×10^5 viable cells (in 30–80 μL) were placed in each well of Falcon 24-well plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) containing 1 mL of medium with 10% FCS, unless stated otherwise. Cells were cultured at 38.5ºC with 95% air 5% CO_2 atmosphere, and the medium was changed approximately every 24 h. During the first 2 d of incubation, cells were cultured in 10% FCS-containing medium, after which the cells were washed twice with serum-free medium (0.5 mL), followed by 24-h hormonal treatments in 0.5 mL of serum-free medium containing 0.25% BSA (to minimize loss of the IGFBPs). Concentrated stock solutions (made in medium) of LH were aliquoted, stored at –20ºC, thawed immediately before use, and further diluted in medium each day of treatment. Insulin was dissolved fresh in culture medium containing 3% 1.0 *N* NaOH and further diluted (1:10^5) in medium. Estradiol was dissolved in 100% ethanol and further diluted in culture medium.

Determination of Numbers of Granulosa and Thecal Cells

After the treatment period had elapsed, medium from each well was collected individually and frozen at (–20ºC) for later use. The number of granulosa and thecal cells was then determined using a Coulter counter (Model Zm; Coulter, Haileah, FL) as previously described *(21,22)*. Briefly, the cells were washed twice with 0.5 mL of 0.15 *M* NaCl followed by the addition of 0.5 mL of trypsin (0.25% [w/v], for 20 min at 25ºC). Following trypsin exposure, the cells were scraped from each well, and the cell aggregates were disrupted via pipetting the cell suspension back and forth

through a 500-µL pipet tip three to five times, diluted in 0.15 *M* NaCl, and enumerated.

Concentration of Medium

To concentrate the IGFBPs, medium samples were ultrafiltered and concentrated approx 10-fold using Centricon concentrators with a mol wt limit of 3000 (Amicon, Beverly, MA) as previously described *(12)*. Briefly, 400 µL of the spent medium was placed inside the sample reservoir of the concentrator and centrifuged at 5322*g* for approx 80 min. The filtrate vial was then discarded, and the sample reservoir was inverted and recentrifuged for 5 min at 591*g* to transfer the retentate into the retentate vial. Final volumes, ranging from 23 to 47 µL, were measured to record the "concentration factor" of the sample; this value was used to correct data as described previously *(12)*.

Gel Electrophoresis of IGFBPs

The concentrated media samples were assessed for IGFBP activity based on molecular weight using one-dimensional, SDS polyacrylamide gel electrophoresis (PAGE) as described previously *(7,12)*. Briefly, 12.5 µL of the concentrated sample was mixed with 12.5 µL of nonreducing Laemmli sample buffer (Bio-Rad, Hercules, CA). After heat treatment (3 min at 100ºC) to denature the proteins, samples were centrifuged at 4657*g* for 3 min and individually added to the 12- or 15-lane SDS-PAGE. Twenty-five microliters of a wide-range color marker (mol wt of 6500–205,000) (Sigma) and a mixture of 4 µL of bovine follicular fluid with 21 µL of the sample buffer were included to identify band size and IGFBPs. The samples were electrophoresed overnight (approx 18–20 h) at constant current (25–30 amps) and varying voltage. Following electrophoresis, the gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific) for 2.5–3.0 h. Each nitrocellulose paper was then blocked using BSA, labeled with ^{125}I-IGF-2 (approx 15,000 cpm/0.1 mL; total volume = 6 mL), and placed on a rocking platform at 4ºC overnight. The next day, the blots were washed in Tris-buffered saline (TBS) with 0.1% Tween followed by additional washings with only TBS. The nitrocellulose blots were then dried and placed on X-ray film for 14 d at –80ºC. At the end of the 14-d period, X-ray films were developed, and individual bands were densitometrically analyzed using Molecular Analyst (Bio-Rad).

Statistical Analyses

Data are presented as the least squares means ± SEM. Each experiment was replicated three times with different pools of thecal and granulosa cells. Each pool of thecal cells was obtained from six to eight follicles from four to eight cows. Each pool of granulosa cells was collected from approx 10–20 ovaries. For each experiment, treatments were applied in triplicate culture wells. Using the general linear model procedures of SAS *(58)*, treatment effects were determined. Specifically, main effects (treatments and experimental replicates) and interactions on dependent variables (i.e., IGFBP production) were analyzed. IGFBP production, which was corrected for final cell numbers and the concentration factor, was expressed as ADU/(10^5 cells·24 h). Specific differences in IGFBP production between treatments were determined using the Fisher's protected least significant difference test if significant treatment effects were observed *(59)*.

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