HORMONAL CONTROL OF INSULIN-LIKE GROWTH FACTOR BINDING-PROTEIN mRNA EXPRESSION IN BOVINE GRANULOSA AND THECA CELLS: QUANTIFICATION BY REAL-TIME RT-PCR

By

JUSTIN L. VOGE

Bachelor of Science

University of Wisconsin-River Falls

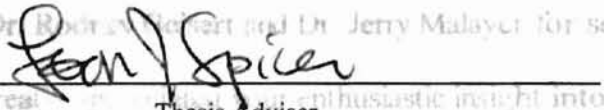
River Falls, Wisconsin

2000

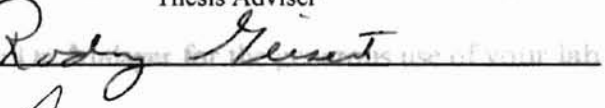
Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
In partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2002

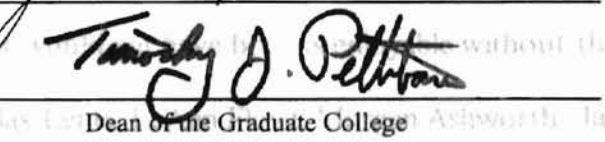
HORMONAL CONTROL OF INSULIN-LIKE GROWTH FACTOR BINDING-PROTEIN mRNA EXPRESSION IN BOVINE GRANULOSA AND THECA CELLS: QUANTIFICATION BY REAL-TIME RT-PCR

Thesis Approved:



Thesis Adviser





Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to start by thanking the members of my committee. First and foremost Dr. Spicer, thank you for affording me the opportunity to continue to my education and enlightening me to the world of research. You have continually challenged me not only to be diligent in learning but to think, I cannot give enough thanks for all the insight and knowledge you have given over the past couple of years.

Thank you also to Dr. Rodney Geisert and Dr. Jerry Malayer for serving on my committee. Dr. Geisert I greatly appreciated your enthusiastic insight into the world of teaching. Also thank you to Dr. Malayer for the generous use of your lab so I could conduct my project.

My time in Stillwater would not have been as enjoyable without the friendship of many graduate students. Clay Lents, LeAnn Floyd, Morgan Ashworth, Jason Ross, Frankie White, Robert Peterson and Jason Schnieder, thank you. I would especially like to thank my officemate LeAnn Floyd, thank you for your friendship, without your expert matchmaking skills I would have never met Julie. Clay Lents, thank you for all your help during my time here, I greatly appreciate it. I would also like to thank Dave Goad, without your help with my project I might still be trying to get things to work. I would also like to thank Dustin Allen for his technical assistance with the ultrasound study and Paula Cinnamon for her expert secretarial assistance.

I would also like to thank my family and especially my parents, Rick and Jane Voge. You have constantly supported me and told me I can go as far as I want. Thank you for listening to me when I am frustrated, praising me when I do well and still reminding me at times I can do better. I love you and thank you.

Last but definitely not least, I would like to dedicate this thesis to my wife, Julie. Julie, I can never tell you how much your love and never-ending support means to me. You are the best thing that has ever happened to me and I love you more than any words will ever describe.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

A loss of reproductive efficiency in animal agricultural systems can result in a substantial decrease in profitability. For example, in dairy cattle the estimated cost for each day open after approximately 100 d in milk is $4 per day (Stevenson, 2000). Therefore, with an average calving interval of 14.5 months, 100 allowed days open and a 285 d gestation period, the average dairy cow in the United States is open for 50 more days than optimal costing about $200. Milk cow numbers in the United States as of July, 2002 were estimated at 9.15 million (NASS, 2002), therefore losses associated with decreased reproductive efficiency are approximately $2 billion per year in the dairy industry. This loss to Oklahoma dairy farmers is estimated to be about $18 million.

A normal 21-day estrous cycle in cattle is associated with a network of key hormonal events, of which an increase in knowledge of how these hormones interact could lead to the development of techniques that would increase reproductive efficiency. Among these hormones are insulin-like growth factor-1 (IGF-1) and IGF-2, which stimulate mitogenesis and steroidogenesis of ovarian cells (Spicer and Echternkamp, 1995). Bound to IGFs are the high-affinity carrier proteins, insulin-like growth factor binding proteins (IGFBPs), which modulate the amount of bioavailable IGFs to the cell. In cattle, levels of IGF-1 remain relatively constant during follicular growth, while IGFBPs vary with low levels in dominant follicles and high levels in large subordinate follicles (Stewart et al., 1996).

Little information is available at present as to how specific hormones, associated with the estrous cycle and ovarian follicular function, regulate the mRNA expression levels of IGFBPs in cattle. Therefore, the objective of this study was to determine the hormonal regulation of IGFBP mRNA levels in cultured bovine granulosa and theca cells using fluorescent real-time quantitative RT-PCR, with specific focus on the effects of estradiol, insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH) and IGFs.

CHAPTER II

REVIEW OF LITERATURE

FUNCTION OF OVARIAN INSULIN-LIKE GROWTH FACTORS (IGFS)

The ovarian IGF system consists of IGF-1 and IGF-2, IGF receptors as well as six IGF binding proteins (IGFBP-1 thru -6). IGF-1 and IGF-2 are related structurally and functionally to insulin and were first purified and sequenced in 1978 (Rinderkneckt and Humbel, 1978a; 1978b). IGF-1 and IGF-2 are single-chain polypeptides, which are highly conserved in regards to their amino acid sequences across species and are produced locally in many tissues, thus allowing for autocrine and paracrine influences (Spicer and Echternkamp, 1995). The actions of IGFs are modulated via high affinity type I and II IGF receptors, part of the tyrosine kinase receptor family, of which IGF-1, IGF-2 and insulin can all bind to each receptor with varying degrees of affinity (for rev. see Jones and Clemmons, 1995; Spicer and Echternkamp, 1995).

Within in the ovary, IGF-1 and IGF-2 serve as paracrine, autocrine and endocrine regulators of ovarian function. For example, IGF-1 is a potent stimulator of mitogenesis in ovine (Monniaux and Pisselet, 1992), bovine (Spicer et al., 1993; Spicer et al., 2002) and porcine (May et al., 1988) granulosa cell cultures. Similarly, IGF-1 acts synergistically with FSH to increase estradiol output via an increase in aromatase activity in many species (Spicer and Echternkamp, 1995; Silva and Price, 2002; Spicer et al., 2002). Furthermore, IGF-1 also increases bovine thecal cell proliferation and androgen production in vitro (Stewart et al., 1995). However, in cattle levels of IGF-1 in follicular

fluid remain relatively constant during the estrous cycle (Echternkamp et al., 1994; de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996).

IGFBP are a family of six IGF specific carrier proteins, which bind IGFs with a higher affinity than their receptors and increase the half-life of IGFs (Jones and Clemmons, 1995; Spicer and Echternkamp et al., 1995; Rajaram et al., 1997). In the bovine ovary, IGF-1 and IGFBP-3 levels remain relatively constant during follicular growth while levels of IGFBP-2, -4 and -5 in follicular fluid fluctuate dramatically such that IGFBP-2, -4 and -5 are nearly absent in bovine dominant ovulatory (Funston et al., 1996; Spicer et al., 2001) and nonovulatory (de la Sota et al., 1996; Stewart et al., 1996; Nicholas et al., 2002) follicles, with higher levels found in large subordinate and small follicles. Moreover, concentrations of estradiol in follicular fluid of cattle are negatively correlated with follicular content of IGFBP-2, -4 and -5 (Stewart et al., 1996; Spicer et al., 2001; Rhodes et al. 2001). Furthermore, IGFBP-2, -3, -4, and -5 inhibit IGF-1-stimulated ovarian function by sequestering IGF-1 (Spicer and Echternkamp, 1995; Spicer et al., 1997; Spicer and Chamberlain, 1999; Brown and Braden, 2001). Thus changes in IGFBP levels due to fluctuating hormonal profiles associated with the estrous cycle may be modifying the amount of bioavialable IGF to bind to its receptor and have an affect on ovarian function. Therefore the aim of this review of the literature was to determine were IGFBP mRNA are localized within in the ovary as well as to how FSH, LH, IGFs, insulin and estradiol effect IGFBP production.

HORMONAL CONTROL OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

IGFBP-1

IGFBP-1 is a 25-34 kDa protein, which has an equal affinity for both IGF-1 and IGF-2 (Spicer and Echternkamp, 1995; Fowler et al., 2000). In the rat (Nakatani et al., 1991) and porcine (Zhou et al., 1996) ovary, localization of IGFBP-1 mRNA expression has not been detected in any tissue as assessed by in situ hybridization. In the human ovary, IGFBP-1 mRNA expression was detectable by in situ hybridization only in granulosa cells of dominant follicles (El-Roeiy et al., 1994). This seems to be a pattern of expression that is associated with preovulatory follicles that are undergoing the luteinization process, as IGFBP-1 mRNA expression was undetectable by either Northern blot analysis or RT-PCR in any compartment of the human ovary before the LH surge (Voutilainen et al., 1996). More recently, immunocytochemical staining studies revealed that IGFBP-1 was found only in human granulosa cells that were luteinized and co-staining for hCG (Gersak et al., 1999). IGFBP-1 mRNA expression in the bovine ovary, in contrast to the human, seems to be an event associated with luteolysis, as an increase in IGFBP-1 expression was seen within 24 to 48 h following prostaglandin-induced luteal regression when compared to controls as detected by differential display RT-PCR (Sayre et al., 2000). However, others have detected a weak signal for IGFBP-1 mRNA in the theca interna of bovine ovaries through RT-PCR (Schams et al., 1999; Schams et al., 2002). Taken together, the ovarian localization and expression of IGFBP-1 mRNA is highly species dependent. Furthermore, an endocrine function of IGFBP-1 has been

postulated in the mouse, as mice overexpressing IGFBP-1 mRNA in the liver have fewer gonadotropin responsive follicles within their ovaries (Froment et al., 2002).

FSH. Emergence of an ovarian follicular cohort, consequent growth followed by selection and attainment of dominance by one follicle in that cohort is associated with a systemic rise and subsequent decline of follicle-stimulating hormone (FSH) in cattle (Ginther et al., 1996; Ginther, 2000). Thus FSH plays an important regulatory role in ovarian follicular growth. This role may in part be through the down-regulation of IGFBPs, allowing for more bioavailable IGF-1 to synergize with FSH to promote positive ovarian function and development. The effects of FSH on ovarian IGFBP-1 synthesis seem to vary depending on the experimental situation. For instance, Mason et al. (1993) found that FSH in low doses (0.1-10 ng/ml) increased IGFBP-1 production in granulosa cells cultured for 48 h from normal and polycystic human ovaries. In rat granulosa cells, low doses (< 10 ng/ml) of FSH increased IGFBP-1 detected in culture medium, while increasing doses up to 100 ng/ml caused a decrease in IGFBP-1 production (Adashi et al., 1990; Adashi et al., 1991). Consistent with this, Dor et al. (1992) found that luteinizing human granulosa cells obtained after in vivo exposure to FSH/hCG had 52% decreased levels of IGFBP-1 compared to controls when cultured for 48 h. However, other studies (Jalkanen et al., 1989; Ovesen et al., 1994) have refuted these results showing no effect of FSH at doses of 10 to 1000 ng/ml for 48 h on human granulosa cell IGFBP-1 production in culture when compared to untreated controls. These differences in part may be explained by differences in culture conditions (i.e.,

duration of exposure to and dose of FSH) as well as cell types (luteinized versus non-luteinized granulosa cells).

LH. The pulsatile secretion of luteinizing hormone (LH) plays a key role in maintenance of the dominant follicle during follicular growth, and secreted in a surge stimulates the ovulatory process (Spicer and Echternkamp, 1986; Ginther et al., 1996). The role that LH plays in regulating ovarian IGFBP production is still not completely known. In cultured human granulosa luteal cells, 100 ng/ml of hCG had no effect on IGFBP-1 production (Jalkanen et al., 1989). Similarly, Voutilainen et al. (1996) reported that 10 ng/ml of LH had no effect on IGFBP-1 gene expression levels in cultured human thecal cells. However, other studies have shown that human luteinized granulosa cells exposed to 10 ng/ml of hCG for 72 h increased IGFBP-1 levels six- to eight-fold over controls with higher doses (100 and 1000 ng/ml) having no effect (Giudice et al., 1991). Recently, Greisen et al. (2002) demonstrated that doses of 1 and 10 ng/ml of LH significantly inhibited IGFBP-1 production by human granulosa luteal cells after 48 h in culture. Therefore these studies would suggest that the effects of LH on IGFBP-1 are associated with dose and/or duration of treatment in vitro.

IGFs. IGFs are potent stimulators of ovarian function, however their role in the regulation of IGFBPs is still not completely known. In human granulosa-luteal cells, IGF-1 and IGF-2 decreased the amounts of IGFBP-1 at doses as low as 0.50 and 1.0 ng/ml, respectively (Dor et al., 1992; Mason et al., 1993; Poretsky et al., 1996). Similarly, IGF-1 as well as IGF-2 significantly decreased both IGFBP-1 protein and

mRNA levels in human endometrial cells (Thrailkill et al., 1990; Irwin et al., 2001). Likewise, IGF-1 lowered IGFBP-1 protein levels in cultured rat hepatocytes (Scharf et al., 1996). In contrast, IGF-1 and IGF-2 increased IGFBP-1 levels in human fetal fibroblasts (Hill et al., 1989). Constant infusion of IGF-1 (4 μg/gBW/d) to immature rats also increased plasma IGFBP-1 levels (Gruaz et al., 1997). The results of these studies suggest that the effects of IGFs on IGFBP-1 production may be dependent on cell type. Because of the possible endocrine effect of IGF-1 on systemic IGFBP-1, the impact on follicular function may depend on the prevalence of the paracrine versus endocrine source of IGFBP-1.

Insulin. Insulin is a potent stimulator of both ovarian mitogenesis and steroidogenesis in several species (see review, Spicer and Echternkamp, 1995). For example, exogenous administration of insulin to cattle increased follicular fluid concentrations of estradiol (Simpson et al., 1994). Insulin appears to be the main modulator of IGFBP-1 gene expression due to the presence of an insulin response element in the promoter region for IGFBP-1 (see review, Fowler et al., 2000). Specifically, insulin decreases both IGFBP-1 mRNA expression levels and gene transcription rates in human and rat hepatoma cells (Powell et al., 1991; Orlowski et al., 1991) by inhibiting IGFBP-1 promoter acitivity (Durham et al., 1999; Tomizawa et al., 2000). In streptozoicin-induced diabetic gilts, levels of IGFBP-1 in serum were increased and conversely IGF-1 concentrations in follicular fluid were decreased (Edwards et al., 1996). In vitro 48 h treatment of human granulosa-luteal cells with 5, 25 or 250 ng/ml (Holst et al., 1997) and 100 ng/ml (Poretsky et al., 1996) of insulin all resulted in a

decline of IGFBP-1 protein accumulation. Greisen et al. (2002) demonstrated that insulin's effect on human granulosa cells was also a dose dependent phenomenon, with insulin at concentrations of 100, 400 and 800 μU/ml significantly reducing IGFBP-1 protein levels to 37, 28 and 24%, respectively of controls. Insulin's action in granulosa cells is not through the classic glucose phosphatidyl-inosital-3 kinase (IP-3) signaling pathway for insulin, as preincubation with wortmannin (a specific inhibitor of IP-3) did not inhibit insulin's negative effect on IGFBP-1 production in human granulosa-luteal cells (Poretsky et al., 2001). Overall, insulin may enhance IGF action via its inhibition of both IGFBP-1 protein and mRNA production.

Estradiol. Concentrations of estradiol found in circulation increase in a parallel fashion with the growth of the dominant follicle as steriodogenic capacity of the granulosa and theca cells increase. IGF-1 is a potent stimulator of both thecal androgen and granulosa cell estradiol production in cattle (Spicer and Echternkamp, 1995; Stewart et al., 1995; Spicer et al., 2002). Similarly, in vivo the loss of estrogenic capability of the dominant non-ovulatory follicle is associated with an increase in IGFBP-2, -4 and -5 levels in follicular fluid of cattle (Funston et al., 1996; Stewart et al., 1996). In vivo administration of estrogen stimulates IGFBP-1 in human serum (Martikainen et al., 1992) and baboon endometrium (Fazleabas et al., 1989). Ovariectomized rats administered estradiol had decreased IGFBP-1 mRNA and protein levels (Molnar and Murphy, 1994). However, estradiol had no effect on IGFBP-1 protein levels in MCF-7 breast cancer cells (Kim et al., 1991). This would suggest that the effect of estradiol on IGFBP-1 is species and cell type specific.

IGFBP-2

IGFBP-2 is a 29-40 kDa binding protein which has a greater affinity for IGF-2 than IGF-1 (Spicer and Echternkamp, 1995). IGFBP-2 mRNA expression using in situ hybridization has been shown in both the theca and granulosa cell layers of bovine ovarian follicles (Armstrong et al., 1998; Yuan et al., 1998). However, expression intensity is less in granulosa cells of large healthy follicles and increases in granulosa and theca cells of atretic and subordinate follicles (Armstrong et al., 1998; Yuan et al., 1998). This is further substantiated in cattle by RT-PCR, as estrogen-active follicles had no detectable IGFBP-2 mRNA in granulosa cells, with increased amounts in theca cells of follicles with decreased estrogen levels (Schams et al., 1999; Schams et al., 2002). IGFBP-2 mRNA expression evaluated with in situ hybridization was most abundant in the granulosa cell layer of porcine small and medium follicles, with decreased amounts present in granulosa cells of preovulatory follicles (Zhou et al., 1996; Liu et al., 2000). In another study, Northern blot analysis indicated that as the follicle progressed in its growth through the estrous cycle IGFBP-2 mRNA expression decreased in granulosa cells of gilts (Samaras et al., 1993). In the human ovary, IGFBP-2 mRNA expression is most prevalent in theca and granulosa cells of small follicles and theca of dominant follicles in studies using both Northern blot (Voutilainen et al., 1996) and in situ hybridization (El-Roeiy et al., 1994). Granulosa cells, but not theca cells of the rat ovary express mRNA for IGFBP-2 (Nakatani et al., 1991). IGFBP-2 mRNA expression is highest in granulosa and theca cells of atretic follicles in the ovine ovary (Besnard et al., 1996). Taken together these studies suggest that localization of IGFBP-2 mRNA is decreased in

healthy, growing follicles and increases during the process of atresia. Furthermore, which cell layer of the follicle expresses IGFBP-2 mRNA may be species dependent.

FSH. FSH (200 ng/ml) inhibited production of IGFBP-2 in porcine granulosa cells from medium-follicles (4-6 mm) (Mondschein et al., 1990). Armstrong et al. (1998) also demonstrated a decrease in IGFBP-2 mRNA, as detected by RT-PCR, in bovine granulosa cells exposed to 50 ng/ml FSH for 4 days. However, other studies have shown that FSH increased IGFBP-2 production by equine granulosa cells (Davidson et al., 2002) and induces its mRNA expression in the ovarian interstitium of mice (Wandji et al., 1998). Recently, Chamberlain and Spicer (2001) reported no effect of FSH (50 ng/ml) on IGFBP-2/-5 production by bovine granulosa cells from small-follicles (1-5 mm). Also treatment with 100 ng/ml of FSH had no significant effect on IGFBP-2 production by human granulosa-luteal cells (Cataldo et al., 1993). These differences in the effects of FSH on IGFBP-2 mRNA expression and protein production may be attributed to different doses of FSH, length of exposure to treatment as well as species.

LH. In cultured rat thecal cells, LH had no effect on neither IGFBP-2 mRNA levels (Voutilainen et al., 1996) nor IGFBP-2 production (Erickson et al., 1995) when exposed for 48 h to doses of 10 ng/ml and 20 uU/ml, respectively. Similarly, LH had no effect in human granulosa-luteal cells (Griesen et al., 2002) or bovine thecal cells (Spicer and Chamberlain, 2002) on IGFBP-2 production in vitro. However, when luteinizing human granulosa cells were exposed to 10 ng/ml of hCG for 72 h in culture a 31 kDa band corresponding to IGFBP-2 increased when compared to untreated controls (Giudice

et al., 1991). In contrast to this, granulosa cells collected from large, more differentiated bovine follicles exposed to 100 ng/ml of LH for 24 h decreased levels of a band that comigrated with IGFBP-2/-5 (Spicer and Chamberlain, 2002). In agreement, IGFBP-2 production was inhibited in both human granulosa cells (Cataldo et al., 1993) and ovine thecal cells by hCG (100 ng/ml) and LH (10 ng/ml), respectively. In vivo, hCG treatment of weaned sows caused a decrease in IGFBP-2 levels in follicular fluid 0-18 h post-administration (Howard and Ford, 1992). These levels remained low for 18-24 h and increased 30-36 h post hCG treatment nearing the time of ovulation (Howard and Ford, 1992). Collectively, these results would suggest that the effects of LH on IGFBP-2 may depend on cell type, length of LH exposure and dose of LH in vitro. Whether increased levels of IGFBP-2 could be used as a marker of luteinization in ovulatory follicles will require further study.

IGFs. In cultured human granulosa cells, treatment with 30 ng/ml of IGF-2 decreased IGFBP-2 levels by 49% when compared to controls (Cataldo et al., 1993). Treatment with [Leu^{27}]IGF-2, an IGF-2 analog which binds only to the type II IGF receptor (Beukers et al., 1991), caused the same result as seen with treatment with IGF-2 (Cataldo et al., 1993). Furthermore, IGF-1 was without effect, leading to the conclusion that the decrease in IGFBP-2 was mediated via the type II IGF receptor in human granulosa cells (Cataldo et al., 1993). However, in cultured rat theca (Erickson et al., 1995), ovine granulosa (Armstrong et al., 1996), and porcine granulosa (Grimes and Hammond, 1992) cells, IGFBP-2 protein levels were increased by treatment with IGF-1. Outside the ovary, treatment with 100 ng/ml of either IGF-1 or IGF-2 for 48 h increased

IGFBP-2 levels in human kidney cells (Boisclair et al., 1994), while 50 ng/ml had no effect in vascular smooth muscle cells (Cohick et al., 1993). In contrast, IGF-1 inhibited IGFBP-2 protein production in rat hepatocytes (Scharf et al., 1996). Therefore, the effects of IGFs on IGFBP-2 may depend on species, cell type as well as the type of IGF receptor present in that cell type.

Insulin. In cultured porcine (Grimes and Hammond, 1992) and human (Greisen et al., 2002) granulosa cells, insulin stimulated production of IGFBP-2 after 48 h of treatment. Boisclair et al. (1994) demonstrated that 48 h exposure to 100 ng/ml of insulin increases IGFBP-2 production in human kidney cells. Furthermore, bovine theca cells cultured for 24 h in serum free medium containing 100 ng/ml of insulin had increased production of IGFBP-2 (Spicer and Chamberlain, 2002). A study with rat intestinal cells revealed that insulin at high (1.0 and 5.0 μg/ml) doses increased and at very high (10.0 μg/ml) doses decreased IGFBP-2 protein levels (Guo et al., 1995). Although IGFBP-2 mRNA levels in the same study were decreased by 5.0 μg/ml of insulin, implying that low doses may promote secretion of stored IGFBP-2 and high doses are inhibiting IGFBP-2 at the genomic level (Guo et al., 1995). Alternatively, the very high doses of insulin may have cross-reacted with IGF-1 receptors exhibiting an IGF-1 effect previously reported for this cell type, as 20 ng/ml of IGF-1 decreased IGFBP-2 mRNA levels compared to untreated controls (Simmons et al., 1995). Similarly, insulin (10^{-7} M) inhibited IGFBP-2 mRNA (Schmid et al., 1992) and protein levels (Schmid et al., 1992; Scharf et al., 1996) in rat hepatocytes. Bovine granulosa cells collected from both small and large follicles had decreased intensity of a large band that comigrated with IGFBP-

2/-5 on a ligand blot after treatment with 100 ng/ml insulin (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002) but whether this band was IGFBP-2, IGFBP-5 or both was not determined. Contrary to these results, equine granulosa cells exposed to 100 ng/ml of insulin for 48 h had no detectable differences in IGFBP-2 production when compared to controls (Davidson et al., 2002). Therefore it seems that regulation of IGFBP-2 by insulin may depend on species, cell type examined as well as length of exposure and dose in vitro.

Estradiol. Estradiol increased the relative abundance abundance of IGFBP-2 mRNA transcripts in the theca-interstitial layer of hypophsectomized rats after 5 days in vivo (Ricciarelli et al., 1991) and in human endometrial cells after 14 days in vitro (Liu et al., 1997). Similarly, equine granulosa cells treated with 500 ng/ml of estradiol for 24 h had increased IGFBP-2 protein levels in culture media (Davidson et al., 2002). However, in equine (Bridges et al., 2002), normal healthy woman (San Roman and Magoffin, 1993), porcine (Howard and Ford, 1992) and bovine (Echternkamp et al., 1994; Funston et al., 1996; Stewart et al., 1996; Austin et al., 2001) follicles, levels of IGFBP-2 in follicular fluid decrease as estradiol levels increase. In cattle these changes in IGFBP-2 in follicular fluid could not be contributed to proteolysis, as little to no cleavage of IGFBP-2 was demonstrated by follicular fluid of dominant follicles (Spicer et al., 2001). Schams et al. (1999 and 2002) demonstrated that levels of IGFBP-2 mRNA expression levels as detected by RT-PCR in thecal cell layer of cattle decreased as estradiol levels increased. In vitro, estradiol had no effect on IGFBP-2 protein in bovine granulosa cells (Spicer and Chamberlain, 2002). These studies taken together suggest that estradiol's

effect on IGFBP-2 may be species as well as cell specific. Furthermore, estradiol is more than likely changing levels of IGFBP-2 through control of its mRNA.

IGFBP-3

IGFBP-3, which binds both IGF-1 and -2 with equal affinity, is a 150 kDa protein that is visualized as a doublet between 40 and 44 kDa on a western ligand blot (Spicer and Echternkamp, 1995). Whether or not IGFBP-3 mRNA expression is localized to a particular ovarian compartment appears to vary between species and technique used. For example, in the bovine ovary in situ hybridization could only detect IGFBP-3 mRNA expression in the granulosa cell layer in 2 of 28 follicles studied (Yuan et al., 1998). Similarly, another study using in situ hybridization detected IGFBP-3 mRNA in the oocyte of preantral follicles and theca externa, but not granulosa cells of cattle (Armstrong et al., 2002). However, using RT-PCR IGFBP-3 mRNA expression was detectable in all follicle classes in both granulosa and theca cells of cattle (Schams et al., 1999; Voge et al., 2001; Schams et al., 2002). In agreement with the latter observations, IGFBP-3 mRNA expression in porcine granulosa cells was constant in all follicles and in theca cells of healthy follicles (Wandji et al., 2000). The acid labile subunit, which forms a ternary complex with IGF-1 and IGFBP-3, was found in preantral and small antral follicle granulosa cells (Wandji et al., 2001). In contrast, others have not detected IGFBP-3 mRNA expression in porcine granulosa cells using in situ hybridization (Zhou et al., 1996) or Northern blot analysis (Samaras et al., 1992). However, the latter study did detect IGFBP-3 mRNA expression in the corpus luteum (CL) and theca cells of the porcine ovary (Samaras et al., 1992). IGFBP-3 mRNA expression was not detectable by Northern blot (Voutilainen et al., 1996), but was detectable by in situ hybridization (El-

Roeiy et al., 1994) in theca cells of small follicles and theca and granulosa cells of large dominant follicles in the human ovary. To date IGFBP-3 mRNA expression has been limited to the CL of the rat ovary (Nakatani et al., 1991). Collectively, IGFBP-3 mRNA is detectable in both theca and granulosa cell compartments of cow, pig and human follicles but sensitive techniques (e.g. RT-PCR) are necessary to detect the low levels of expression.

FSH. Exogenous FSH administered to cattle had no effect on the amount of IGFBP-3 found in follicular fluid of large follicles (Echternkamp et al., 1994). Similarly, no change was seen in follicular IGFBP-3 levels during endogenous changes in serum FSH of cattle (Austin et al., 2001) and FSH had no influence on IGFBP-3 production in small-follicle granulosa cells after 24 h of treatment (Chamberlain and Spicer, 2002). In contrast, Ingman et al. (2000) reported that bovine granulosa cells associated with the oocyte-cumulus complex in small follicles showed an increase in IGFBP-3 production in vitro after 24 h exposure to FSH (10 ng/ml). IGFBP-3 production by granulosa cells cultured from small (1-5 mm) porcine follicles (Mondschein et al., 1990) and human polycystic (San Roman and Magoffin, 1992) ovaries was attenuated by exposure to FSH. These data taken together would suggest that the granulosa cell response to FSH on IGFBP-3 production may be species dependent but also may be influenced by culture conditions.

LH. In vivo levels of IGFBP-3 remained constant among follicles both before and after the ovulatory surge of LH in cattle (Funston et al., 1996). Similarly, treatment with LH had no effect on IGFBP-3 protein levels in human (Giudice et al., 1991; Greisen et al., 2002) or bovine granulosa cells (Spicer and Chamberlain, 2002) as well as having no effect on human thecal cell IGFBP-3 mRNA levels (Voutilainen et al., 1996). However, in vivo administration of 500 IU of hCG to sows resulted in a decrease in follicular fluid IGFBP-3 from 0 to 36 h but decreases in plasma IGFBP-3 likely accounted for the intrafollicular changes (Howard and Ford, 1992). Treatment of human thecal cells with 100 ng/ml of hCG for 48 h blocked production of IGFBP-3 (San Roman and Magoffin, 1992). In contrast, LH increases IGFBP-3 mRNA thecal cells from rats (Erickson et al., 1995) and increases IGFBP-3 production in theca cells from cattle (Spicer and Chamberlain, 2002). Thus the role of LH in regulating IGFBP-3 may depend on species as well as cell type examined. Interestingly, Fraser et al. (2000) demonstrated that administration of hCG to late-luteal human patients, in order to rescue the CL from luteolysis, resulted in an increase in IGFBP-3 mRNA levels in endothelial cells of the CL similar to early-luteal levels. High IGFBP-3 mRNA expression was identified in endothelial cells of microvessels of the primate CL (Fraser et al., 1998). Whether IGFBP-3 production by theca cells is due to possible contaminating endothelial cells remains to be determined.

IGFs. In human luteinized granulosa cells treatment with IGF-1 and IGF-2 stimulated IGFBP-3 release by at least 3-fold in a dose-dependent manner (Cataldo et al., 1993). Similarly, Grimes and Hammond (1992) demonstrated that IGF-1 treatment for

24 h increased IGFBP-3 protein and mRNA levels in moderately differentiated porcine granulosa cells. Furthermore, treatment with 50 ng/ml of IGF-1 for 24 h further stimulated an FSH-induced increase in IGFBP-3 levels in bovine granulosa cells (Ingman et al., 2001). In other cell types, treatment with IGF-1 caused an increase in IGFBP-3 protein concentrations in human (Camacho-Hubner et al., 1992) as well as bovine (Bale and Conover, 1992) fibroblasts by 2- and 6-fold, respectively. Conover et al. (1993) further illustrated that treatment with 10 nM of IGF-2 for 24 h was also able to stimulate IGFBP-3 production in human fibroblasts. IGF-1 also stimulates IGFBP-3 mRNA synthesis in bovine endothelial cells (Eronder et al., 1997). In agreement, both IGFBP-3 mRNA and protein levels were increased in a dose- and time-dependent manner, with this effect first observed at 3 h and plateuing at 24 h of treatment in human osteosarcoma cells (Rosato et al., 2001). Overall in most cell types examined, the role of IGFs is to stimulate production and release of IGFBP-3. Since IGFBP-3 increases the half-life of IGFs, IGF-1-induced increases in local production of IGFBP-3 could provide a local "reserve" of IGF-1.

Insulin. Overall the effects of insulin on IGFBP-3 production seem to vary among both species and cell type. In human granulosa-luteal cells, 2000 ng/ml of insulin for 3 to 5 d was without effect on IGFBP-3 production (Cataldo et al., 1993). This was further confirmed by a lack of effect of 100 ng/ml of insulin on IGFBP-3 protein levels in granulosa and thecal cells collected from large bovine follicles cultured for 24 h (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002). Similarly, IGFBP-3 mRNA expression was not changed by addition of insulin to medium of cultured human

retinal pigment epithelial cells (Feldman and Randolph, 1994). In contrast, porcine granulosa cells obtained from medium (4 to 6 mm) sized follicles had increased IGFBP-3 protein levels after treatment with insulin at concentrations of 17 nM or greater for 48 h (Grimes and Hammond, 1992). In vivo, systemic levels of IGFBP-3 and insulin are positively correlated in gilts (Edwards et al., 1996), humans (Ripa et al., 2002) and cattle (Cohick et al., 1992). Thus, intrafollicular changes in IGFBP-3 may reflect changes in systemic IGFBP-3 under certain situations.

Estradiol. Thecal-interstitial cell mRNA expression for IGFBP-3 was decreased by exogenous administration of estrogens to hypopysectomized rats (Ricciarelli et al., 1992). Spicer and Chamberlain (2002) demonstrated that 500 ng/ml of estradiol had no effect on bovine thecal (large follicles) or granulosa (small and large follicles) cell IGFBP-3 production after 24 h exposure in vitro. Likewise, estradiol had no effect on IGFBP-3 protein levels in porcine granulosa cells (Mondschein et al., 1990) or in cultured human fibroblasts (Camacho-Hubner et al., 1992). Outside the ovary, estradiol had an inhibitory effect on IGFBP-3 mRNA and protein in cultures of human endometrial cells (Liu et al., 1997) as well as decreasing basal levels of IGFBP-3 protein by 25% in MCF-7 breast cancer cells (Martin et al., 1995). Estradiol also increased amounts of IGFBP-3 produced by primary cultures of rat osteoblasts (Schmid et al., 1989). In estrogen active follicles of normal women, IGFBP-3 levels decreased as these follicles grew (San Roman and Magoffin, 1993). In mares, follicular fluid IGFBP-3 levels increase with follicular development (Bridges et al., 2002) However, in follicular fluid of cattle, levels of IGFBP-3 remain constant (de la Sota et al., 1996; Stewart et al., 1996).

Because exogenous estradiol administered to ovariectomized cattle increases serum IGFBP-3 levels, via increased growth hormone levels (Simpson et al., 1997), systemic changes in IGFBP-3 may account for intrafollicular changes in IGFBP-3. The differences among studies as to the influence of estradiol on IGFBP-3 in vitro production may be in part attributed to species differences, dose and duration of treatment as well as cell type.

IGFBP-4

Approximately 24 kDa in size, IGFBP-4 binds both IGF-1 and IGF-2 with an equal affinity (Spicer and Echternkamp, 1995). In the bovine ovary as assessed by in situ hybridization, IGFBP-4 mRNA expression was found to be localized to theca cells of healthy antral follicles and granulosa cells of atretic follicles (Armstrong et al., 1998). However, RT-PCR detected IGFBP-4 mRNA expression in granulosa and theca cells regardless of the follicles estrogenic capacity (Schams et al., 1999; Schams et al., 2002). In the ovine ovary IGFBP-4 mRNA was localized in theca cells of both healthy and atretic follicles examined by in situ hybridization (Besnard et al., 1996). Similar to the bovine, IGFBP-4 mRNA expression in the porcine ovary was found to be greatest in theca cells of growing follicles and granulosa cells of atretic follicles using in situ hybridization (Zhou et al., 1996). Liu et al. (2000) found IGFBP-4 mRNA expression using in situ hybridization in both granulosa and theca cells, with the greatest abundance found in theca cells of large porcine follicles. In humans, IGFBP-4 mRNA expression is located in both granulosa and theca cells using Northern Blot analysis (Vouitalainen et al., 1996) and in situ hybridization (El-Roeiy et al., 1994) techniques. Rat ovarian

IGFBP-4 mRNA expression appears to be confined to granulosa cells of atretic antral and preantral follicles (Nakatani et al., 1991; Erickson et al., 1992). Whether or not IGFBP-4 mRNA expression in granulosa cells can be used as a marker of follicular health requires further elucidation of the mechanisms controlling its expression.

FSH. Grimes et al. (1994) reported that FSH stimulation of porcine granulosa cells from follicles 4 to 6 mm in size increased IGFBP-4 levels in a dose-dependent manner. However, the same study also found no effects of FSH on IGFBP-4 mRNA expression in the same cell type (Grimes et al., 1994). In the rat, IGFBP-4 mRNA has been shown to be localized to granulosa cells of antral follicles and this signal was absent during the proestrus (high FSH) period of preovulatory follicles (Erickson et al., 1992). Another study revealed that low doses (3 ng/ml) of FSH increased while high doses (30 and 100 ng/ml) inhibited IGFBP-4 production by rat granulosa cells (Erickson et al., 1994). Similarly, rat granulosa cells had decreased levels of IGFBP-4 protein and elimination of detectable IGFBP-4 mRNA after 72 h treatment with FSH (Liu et al., 1993). However, Chamberlain and Spicer (2001) found that 24 h exposure to 50 ng/ml of FSH had no impact on IGFBP-4 production in bovine granulosa cells collected from small follicles (1 to 5 mm).

In FSH-treated rat granulosa cell cultures, two molecular weight bands (21.5 and 17.5 kDa) were detected with an IGFBP-4 antibody indicating the possibility of an FSH induced IGFBP-4 protease (Liu et al., 1993). Recently, this protease has been identified as pregnancy associated plasma protein-A (PAPP-A) in both humans (Conover et al., 2001) and farm animal species (Mazerbourg et al., 2001). Taken together these data

suggest that decreases in IGFBP-4 protein may be due to an increase in protease activity as well as a decrease in IGFBP-4 mRNA levels.

LH. Stimulation of both ovine (Armstrong et al., 1996) and bovine (Armstrong et al., 1998) thecal cells with LH (10 and 100 ng/ml, respectively) resulted in an increase of IGFBP-4 protein and mRNA in each, respectively. Similarly, human granulosa-luteal cells exposed to 10 ng/ml of hCG for 72 h had increased levels of IGFBP-4 present in culture medium (Giudice et al., 1991). In vivo, PMSG primed rats had a 3.5-fold increase in IGFBP-4 mRNA expression levels by 24 h post administration of 15 IU of hCG (Putowski et al., 1997). However, Liu and Ling (1993) demonstrated that LH blocked the increase of IGFBP-4 production in a dose-dependent manner in rat granulosa cells. In both small- and large-follicle granulosa (but not theca) cells collected from cattle, LH was able to decrease IGFBP-4 production after 24 h of treatment (Spicer and Chamberlain, 2002). In porcine follicles, levels of LH receptor mRNA were increased whereas IGFBP-4 mRNA levels were decreased in 2 to 6 mm versus 8 mm follicles, suggesting that LH may suppress IGFBP-4 mRNA expression (Liu et al., 2000). To further substantiate this, preovulatory follicles in proestrous (after the FSH/LH surge) rats had no detectable signal for IGFBP-4 mRNA (Erickson et al., 1992). Whether LH, like FSH, induces an IGFBP-4 protease, remains to be determined but may account for the differential/opposing effects of LH on levels of IGFBP-4 protein and mRNA previously reported.

IGFs. The role of IGFs in the regulation of IGFBP-4 seems to vary with cell type. In moderately differentiated porcine granulosa cells, IGF-1 had no effect on IGFBP-4 protein or mRNA concentrations in vitro (Grimes et al., 1994). Cultured rat hepatocytes differed in that 100 nM of IGF-1 increased IGFBP-4 production when compared to controls (Scharf et al., 1996). However, in mouse granulosa cells, 48 h exposure to IGF-1 decreased levels of IGFBP-4 in culture medium (Adashi et al., 1997). Similarly, treatment with both IGF-1 and IGF-2 caused a decrease in IGFBP-4 protein in human endometrium (Irwin et al., 1995) and human fibroblasts (Conover et al., 1993). This decrease was likely mediated through an increase in protease activity as detection of a 16-kDa band that bound immunoreactive IGFBP-4 increased at the same time (Irwin et al., 1995). In rat thecal (Erickson et al., 1995), porcine vascular smooth muscle (Cohick et al., 1993) and rat myometrial cells (Huynh, 2000) cells, treatment with IGF-1 increased IGFBP-4 mRNA expression levels. However, in these same studies, levels of IGFBP-4 protein were decreased by IGF-1 treatment, further substantiating the role for IGFBP-4 protease activity. The role of IGF-1 may be to bind IGFBP-4, thus exposing the cleavage site of IGFBP-4 to the protease. For example, when IGF-1 was added to rat granulosa cell cultures it activated the proteolysis of radiolabelled IGFBP-4 (Iwashita et al., 1998). Furthermore, addition of LR3-IGF-1, which does not bind IGFBP-4, did not result in cleavage of IGFBP-4 further indicating that IGF-1 must bind IGFBP-4 to expose its cleavage site to the protease (Iwashita et al., 1998). Similarly, Mazerbourg et al. (2000) demonstrated that addition of IGFBP-2 and -3 to culture decreased IGFBP-4 proteolysis, though this was reversed upon addition of IGF-1 and IGF-2. As mentioned earlier, in humans (Conover et al., 2001) as well as farm animal species (Mazerbourg et al., 2001),

IGFBP-4 cleavage has been attributed to PAPP-A, an IGFBP-4 specific protease whose proteolytic activity depends on IGF-1 binding IGFBP-4. Therefore, the role of IGFs in IGFBP-4 production may be to stabilize the mRNA, providing a constant source of IGFBP-4 protein, with decreasing protein levels found in media attributed to an increase in bound IGFs exposing the cleavage site for increased IGFBP-4 protease activity (Huynh, 2000).

Insulin. Recently, treatment of bovine small-follicle granulosa cells with 100 ng/ml of insulin for 24 h proved inhibitory to IGFBP-4 production (Spicer and Chamberlain, 2002). However, insulin was without affect on IGFBP-4 production in theca or granulosa cells of large bovine follicles (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002). In human granulosa cells, insulin inhibited IGFBP-4 accumulation in culture medium (Greisen et al., 2002). Outside the ovary, Irwin et al. (1995) demonstrated that insulin had a dose-dependent effect in human endometrial stromal cells, with doses of less than 10 ng/ml and that of 100 ng/ml being without effect and stimulatory to IGFBP-4 production, respectively. Insulin was also stimulatory to IGFBP-4 mRNA and protein levels in porcine smooth vascular smooth muscle cells (Cohick et al., 1993) as well as IGFBP-4 protein levels in human fibroblasts (Camacho-Hubner et al., 1992). In contrast, insulin was without effect when applied to neuroblastoma (Babajko et al., 1997) or fibroblasts (Conover et al., 1993). These studies suggest that insulin's effect on IGFBP-4 may be a dose as well as species and cell specific phenomenon.

Estradiol. Until recently little has been known as to the direct effects of estradiol on IGFBP-4 production in the bovine ovary. Spicer and Chamberlain (2002) demonstrated that 500 ng/ml of estradiol for 24 h significantly decreased bovine small- and large-follicle granulosa cell IGFBP-4 production in vitro. Similarly, the future dominant follicle in the majority of ovarian follicular cohorts of cattle had the highest levels of estradiol and lowest levels of IGFBP-4, when compared to future subordinate follicles (Mihm et al., 2000). Some of this decrease may in part be due to proteolysis, as cleavage of IGFBP-4 by follicular fluid of cattle was positively correlated with estradiol levels in cattle (Spicer et al., 2001). In other tissues, such as human osteoblasts (Kassem et al., 1996) and MCF-7 human breast cancer cells (Qin et al., 1999) estradiol treatment increases IGFBP-4 protein and (or) mRNA levels. Similarly, adminstration of estradiol to ovariectomized rats increased uterine expression levels of IGFBP-4 mRNA (Molnar and Murphy, 1994). However, estradiol was without effect on IGFBP-4 protein in human fibroblasts (Conover et al., 1993) or its mRNA in the hypophysectomized rat ovary (Putowski et al., 1997). This taken together would suggest that the effects of estradiol on IGFBP-4 may depend on cell type and species examined and (or) the production of an IGFBP-4 protease in that cell type.

IGFBP-5

IGFBP-5 is 29-31 kDa protein, which binds IGF-2 to a higher degree than IGF-1 (Spicer and Echternkamp, 1995). To date, few studies have examined the localization of IGFBP-5 mRNA expression in the ovary. IGFBP-5 mRNA expression was detected with

RT-PCR in both granulosa and theca cells in the bovine ovary, with granulosa cell IGFBP-5 mRNA levels decreasing as follicular estradiol levels increased (Schams et al., 1999; Schams et al., 2002). In situ hybridization revealed IGFBP-5 mRNA expression to be associated with granulosa cells of atretic follicles and theca cells of healthy follicles in sheep (Besnard et al., 1996). El-Roeiy et al. (1994) demonstrated the localization of IGFBP-5 mRNA expression in theca, granulosa and stroma of all human ovarian follicles. However, only theca cells from human ovaries had detectable IGFBP-5 mRNA expression when examined by Northern Blot analysis (Voutilainen et al., 1996). In the porcine ovary, IGFBP-5 mRNA was not detected in ovarian follicles, but was in the capillaries of luteal tissue (Zhou et al., 1996). In the rat ovary, antral follicles are devoid of IGFBP-5 mRNA expression, while atretic preantral follicles are the main source of IGFBP-5 mRNA expression detected by in situ hybridization (Erickson et al., 1992; Putowski et al., 1995). Whether or not local production of IGFBP-5 is contributing to follicular fluid levels of IGFBP-5, and what hormonal mechanisms are controlling ovarian IGFBP-5 production needs further study in bovine as well as other species.

FSH. Synthesis of IGFBP-5 by bovine (Chamberlain and Spicer, 2001; Ingman et al., 2000) and ovine (Armstrong et al., 1996) granulosa cells was not affected by treatment with FSH. However, IGF-I-stimulated production of IGFBP-5 protein and mRNA in cultured porcine granulosa cells was dramatically reduced by concurrent treatment with FSH (Grimes et al., 1994). Similarly, that stimulation of rat granulosa cells with high doses of FSH (100 ng/ml) reduces a 28-29 kDa protein corresponding to IGFBP-5 (Adashi et al., 1993; Fielder et al., 1993). Liu et al. (1993) also demonstrated

that both IGFBP-5 protein and mRNA levels, as detected by ligand blotting and Northern blot analysis respectively, are reduced in cultured rat granulosa cells after exposure to FSH conditioned media. Some of this reduction in IGFBP-5 may be due to an FSH-induced protease, as incubation with medium from FSH-treated rat granulosa cells degraded exogenously added IGFBP-5 (Liu et al., 1993) and follicular fluid from dominant follicles of cows (Spicer et al., 2001) and mares (Bridges et al., 2002) contain IGFBP-5 protease activity. This suggests that FSH may regulate granulosa cell IGFBP-5 production through both changes in gene transcription and post-transcriptionally by induction of a protease in ovarian granulosa cells.

LH. In vivo, numbers of thecal LH/hCG binding sites were negatively correlated with IGFBP-5 protein concentrations in follicular fluid of cattle (Stewart et al., 1996). In vitro, IGFBP-2/-5 production was decreased by 100 ng/ml of LH in bovine granulosa cells isolated from large-follicles (Spicer and Chamberlain, 2002), whereas bovine thecal IGFBP-5 production was not affected by LH (Spicer and Chamberlain, 2002). However, Voutilainen et al. (1996) reported that LH increased IGFBP-5 mRNA levels in cultured human thecal cells. These results suggest that species and cell type may influence the effect of LH on IGFBP-5 production within the follicle, and that LH may play a regulatory role in follicular IGFBP-5 production in cattle and humans.

IGFs. Moderately differentiated porcine granulosa cells treated with IGF-1 for 48 h increased IGFBP-5 protein and mRNA expression in vitro (Grimes et al., 1994). Likewise, the addition of 100 ng/ml of IGF-1 to rat granulosa cells for 72 h significantly

increased IGFBP-5 protein levels (Fielder et al., 1993). Fielder et al. (1993) also demonstrated that IGF-1 was able to block an FSH induced decrease in IGFBP-5. Furthermore, treatment with IGF-1 was found to be both dose- and time-dependent in rat granulosa cells such that stimulation of IGFBP-5 concentrations as analyzed by western ligand blotting were first detectable at a dose of 1 ng/ml, continuing to increase in intensity up to 100 ng/ml of IGF-1 (Adashi et al., 1994). The time-dependent increases in IGFBP-5 induced by IGF-1 were first detected at 24 h and continued to increase until termination of the experiment at 72 h (Adashi et al., 1994). The effects if IGF-1 are thought to be mediated via the type I IGF receptor alone, as 10 and 100 ng/ml of des(1-3) IGF-1, an IGF-1 analog which binds IGFBP with a lower affinity, was a more potent stimulator of IGFBP-5 accumulation than IGF-1 (Adashi et al., 1994). To further substantiate this, insulin was only effective at doses of 10,000 ng/ml, which likely cross-react with the type I IGF receptor (Adashi et al., 1994). Outside the ovary in cultured human osteoblasts, addition of 0.5 and 10 nM IGF-1 caused a dose dependent increase in IGFBP-5 protein levels by 2.6- and 10-fold, respectively (Conover and Kiefer, 1993). However, these changes in IGFBP-5 protein levels were not accompanied by similar changes in mRNA expression (Conover and Kiefer, 1993). Similarly, in human fibroblasts addition of IGF-1 and IGF-2 caused a 6- to 8-fold increase in IGFBP-5 protein levels, while increasing IGFBP-5 mRNA levels by only 43% (Camacho-Hubner et al., 1992). IGF-1 may regulate IGFBP-5 levels posttranscriptionally by protecting it from IGFBP-5 proteolytic activity, as the proteolytic breakdown of IGFBP-5 decreased upon the addition of IGF-1 to rat granulosa cell culture (Fielder et al., 1993). This protective action of IGF-1 on IGFBP-5 breakdown was further substantiated in ovine articular

chondrocytes, as radiolabelled IGFBP-5 was not significantly degraded when 100 ng/ml of IGF-1 was added to culture (Sunic et al., 1998). However, treatment with 100 ng/ml of Des(1-3)IGF-1 and LR^3IGF-1, which do not bind IGFBPs, resulted in a significant reduction in IGFBP-5 exposed to chondrocyte conditioned medium (Sunic et al., 1998). This IGF-1-induced decrease in proteolysis of IGFBP-5 is the opposite of its effect on IGFBP-4 proteolysis, as PAPP-A proteolysis of IGFBP-4 is increased by IGF-1 (Iwashita et al., 1998; Mazerbourg et al., 2000; Mazerbourg et al., 2001). Furthermore, IGFBP-5 may serve to increase stores of IGFs around the cell surface as it is known to be associated with the extra cellular matrix in bovine granulosa cells (Ingman et al., 2000).

Insulin. Until recently little information has been available as how insulin directly regulates ovarian IGFBP-5 production. In bovine thecal cells treated with 100 ng/ml of insulin for 24 h, IGFBP-5 production was significantly increased (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002). In contrast, the same dose of insulin attenuated production of a large band that comigrated with IGFBP-2/-5 in granulosa cells from small and large bovine follicles (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002), but whether this was IGFBP-2, IGFBP-5 or both was not determined. Furthermore, insulin was without effect on IGFBP-5 production in equine granulosa cells (Davidson et al., 2002) and bovine aortic endothelial cells (Dahlfors and Arnqvist, 2000). Thus the role of insulin in regulating IGFBP-5 production may depend on cell type as well as species. Overall insulin may be having a positive affect on bovine granulosa cell function by decreasing IGFBP-5 levels allowing for more free IGF-1 to be available to its receptor.

Estradiol. Levels of a molecular weight band that co-migrated with IGFBP-2/-5 on a ligand blot decreased in bovine large-follicle granulosa cells after treatment with estradiol for 24 h (Spicer and Chamberlain, 2002). However, the same study found no effect on either thecal or small-follicle granulosa cell IGFBP-5 production. Consistent with this, equine granulosa cells exposed to 500 ng/ml of estradiol for 24 h had no effect on IGFBP-5 production (Davidson et al., 2002). Similarly, estradiol evoked no change in IGFBP-5 protein production in osteoblasts (Conover and Kiefer, 1993). However, levels of IGFBP-5 are lower in follicular fluid of dominant versus subordinate follicles in cattle (Stewart et al., 1996; Spicer et al., 2001). As mentioned earlier, some if these changes may be attributed to proteolysis, as follicular fluid from large estrogen-active follicles in horses (Bridges et al, 2002) and cattle (Spicer et al., 2001) cleaved IGFBP-5. Therefore, changes in IGFBP-5 in follicular fluid may be in part due to an increase in proteolysis of IGFBP-5 and a decrease in its granulosa cell production. Recently, Overgaard et al. (2001) characterized an IGFBP-5 specific metalloproteinase called PAPP-A2 from mammalian cells, which cleaved IGFBP-5 but not IGFBP-4. Whether the majority of IGFBP-5 proteolysis is due to the IGFBP-5 specific protease, PAPP-A2 (Overgaard et al., 2001), or some other IGFBP proteases such as PAPP-A (Laursen et al., 2001) or kallikrein K2 (Rehault et al., 2001) in the ovarian follicle will require further study.

IGFBP-6

IGFBP-6 is a 21-32 kDa protein that was first identified in adult serum and binds IGF-2 with a greater affinity than IGF-1 (Spicer and Echternkamp, 1995). In the bovine

ovary IGFBP-6 mRNA expression was detected in both granulosa and theca cells using RT-PCR (Schams et al., 1999; Schams et al., 2002). Similarly, human ovaries contained IGFBP-6 mRNA expression in granulosa and theca cells as detected with RT-PCR (Voutilainen et al., 1996) but not with in situ hybridization (El-Roeiy et al., 1994). Rat ovarian IGFBP-6 mRNA expression was detected by ribonuclease protection assay and is limited to the theca cell layer (Rohan et al., 1993).

FSH. IGFBP-6 transcripts have been found to be expressed in the ovaries of PMSG-primed rats (Shimaski et al., 1991). Hypophysectiomized rats have a 2.3-fold greater whole ovarian IGFBP-6 gene transcpripts than intact controls (Rohan et al., 1993). When exogenous FSH was given back to these animals it resulted in a 2.4-fold decrease in IGFBP-6 compared to non-treated hypophysectomized animals (Rohan et al., 1993). However, IGFBP-6 gene expression was limited to the theca cell layer, suggesting that a granulosa cell intermediate stimulated by FSH was decreasing thecal IGFBP-6 gene transcription (Rohan et al., 1993).

LH. Little to no work has been done as to the effects of LH on ovarian IGFBP-6 production. Voutilainen et al. (1996) demonstrated IGFBP-6 mRNA expression in ovarian tissue using RT-PCR, however this signal was not present in cultured theca cells treated with LH as detected by Northern Blot analysis.

IGFs. To date, no data are available as to the affect of IGF-1 or IGF-2 on IGFBP-6 production in the ovary. However, neither IGF-1 nor IGF-2 were able to

influence IGFBP-6 mRNA expression levels in rat osteoblasts (Gabbitas and Canalis, 1997). Furthermore, levels of IGFBP-6 mRNA transcripts were not affected by addition of IGF-1 to culture medium of human retinal pigment epithelial cells (Feldman and Randolph, 1994). However, in neuroblastoma cells treatment with IGF-1 induced IGFBP-6 protein production and detection of its mRNA by RT-PCR (Babajko et al., 1997). Taken together these studies suggest the effect of IGFs on IGFBP-6 mRNA and protein levels may depend on cell type studied.

Insulin. To date no information is available on insulin regulation of IGFBP-6 in any ovarian cell type. In retinal pigment epithelial cells (Feldman and Randolph, 1994) and neuroblastoma cells (Babajko et al., 1997), insulin was unable to influence IGFBP-6 protein or mRNA expression levels. Insulin does not seem to be as potent a modulator of IGFBP-6 production when compared to other IGFBPs.

Estradiol. Limited information is available as to the effects of estradiol on IGFBP-6. Treatment of hypophysectomized rats with diethylstilbestrol (DES) caused an approximate 2.5-fold decrease in whole ovarian transcripts of IGFBP-6 (Rohan et al., 1993). This was further attenuated by treatment of DES + FSH, suggesting that a granulosa cell intermediate was causing this decrease (Rohan et al., 1993). However, in MCF-7 cells treatment with 10 nM of estrogen increased IGFBP-6 by 1.5- to 2-fold after 96 h versus untreated controls (Martin et al., 1995).

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION: SELECTION OF AN INTERNAL CONTROL

Survival, growth and differentiation of cells is often reflected in different patterns of gene expression. For this reason, the importance of being able to quantitate mRNA transcription levels of specific genes has been extremely important in many areas of research today. Many techniques are used to accomplish this goal and among them are quantitative real-time RT-PCR, northern blot analysis, RNAse protection assays and in situ hybridization. However, errors in the quantitation of mRNA transcripts are easily compounded by any variation in starting material between samples, which can lead to false differences in quantification of a particular gene. Therefore, a housekeeping or normalizing gene is needed to correct for loading variation of the target gene of interest. Housekeeping genes are defined as a cellular RNA that is expressed at constant levels among different tissues, at all stages of development and should be unaffected by experimental treatments (Bustin, 2000). Suzuki et al. (2000) reported that the three most common genes used for this purpose are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and 18S rRNA. Many studies have looked if the experimental paradigm affects expression of these genes. For instance, in serum stimulated fibroblasts serum increased the total amount of mRNA found in these cells while not increasing the amount of total RNA (Schmittgen and Zakrajsek, 2000). When not normalized for RNA content serum significantly increased all three housekeeping genes expression over time (Schmittgen and Zakajsek, 2000). However, when normalized for RNA content 18S rRNA did not change while GAPDH and β-actin increased over time exposed to serum (Schmittgen and Zakajsek, 2000). Cultured alveolar macrophages varied more in β-actin expression levels than in GAPDH when exposed to various cell stimulators in culture

over a 22 h period (Foss et al., 1999). During differentiation, cultured brown adipocytes increased in GAPDH mRNA expression when compared to 18S rRNA over a 5 d period with exposure to insulin increasing this effect when included in the culture (Barroso et al., 1999). This increase in GAPDH mRNA levels due to insulin occurred in a time dependent manner, increasing over a 20 h period (Barroso et al, 1999). Insulin, IGF-1 and IGF-2 in cultured adipocytes also caused a dose dependent increase in GAPDH expression levels when exposed to each for 24 h (Barrosos et al., 1999). These findings suggest that the experimenter must first validate a housekeeping gene as it pertains to their experimental paradigm before using it as an internal control. For the purposes of this thesis, 18S rRNA was chosen due to it lack of response to serum stimulation as well as the hormones insulin, IGF-1 and -2 all of which's effects were tested in culture. Therefore, with each of the previous hormones differentially controlling the production of protein and mRNA for a particular IGFBP, the objective of this thesis is to determine the hormonal control of IGFBP-2, -3 and –4 mRNA expression in bovine granulosa and theca cells.

LITERATURE CITED

Adashi, E. Y., C. E. Resnick, E. R. Hernandez, A. Hurwitz, and R. G. Rosenfeld. 1990. Follicle-stimulating hormone inhibits the constitutive release of insulin-like growth factor binding proteins by cultured rat ovarian granulosa cells. Endocrinology 126:1305-7.

Adashi, E. Y., C. E. Resnick, A. Hurwitz, E. Ricciarelli, E. R. Hernandez, and R. G. Rosenfeld. 1991. Ovarian granulosa cell-derived insulin-like growth factor binding proteins: modulatory role of follicle-stimulating hormone. Endocrinology 128:754-60.

Adashi, E. Y., C. E. Resnick, D. W. Payne, R. G. Rosenfeld, T. Matsumoto, M. K. Hunter, S. E. Gargosky, J. Zhou, and C. A. Bondy. 1997. The mouse intraovarian insulin-like growth factor I system: departures from the rat paradigm. Endocrinology 138:3881-90.

Adashi, E. Y., C. E. Resnick, and R. G. Rosenfeld. 1994. IGF-I stimulates granulosa cell-derived insulin-like growth factor binding protein-5: evidence for medication via type I IGF receptors. Mol Cell Endocrinol 99:279-84.

Adashi, E. Y., C. E. Resnick, C. Tedeschi, and R. G. Rosenfeld. 1993. A kinase-mediated regulation of granulosa cell-derived insulin-like growth factor binding proteins (IGFBPs): disparate response sensitivities of distinct IGFBP species. Endocrinology 132:1463-8.

Armstrong, D. G., G. Baxter, C. O. Hogg and K. J. Woad. 2002. Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. Reproduction 123: 789-97.

Armstrong, D. G., G. Baxter, C. G. Gutierrez, C. O. Hogg, A. L. Glazyrin, B. K. Campbell, T. A. Bramley, and R. Webb. 1998. Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinology 139:2146-54.

Armstrong, D. G., C. O. Hogg, B. K. Campbell, and R. Webb. 1996. Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. Biol Reprod 55:1163-71.

Austin, E. J., M. Mihm, A. C. Evans, P. G. Knight, J. L. Ireland, J. J. Ireland, and J. F. Roche. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 64:839-48.

Babajko, S., P. Leneuve, C. Loret, and M. Binoux. 1997. IGF-binding protein-6 is involved in growth inhibition in SH-SY5Y human neuroblastoma cells: its production is both IGF- and cell density-dependent. J Endocrinol 152:221-7.

Bale, L. K. and C. A. Conover. 1992. Regulation of insulin-like growth factor binding protein-3 messenger ribonucleic acid expression by insulin-like growth factor I. Endocrinology 131:608-14.

Barroso, I., B. Benito, C. Garci-Jimenez, A. Hernandez, M. J. Obregon, and P. Santisteban. 1999. Norepinephrine, tri-iodothyronine and insulin upregulate glyceraldehyde-3-phosphate dehydrogenase mRNA during brown adipocyte differentiation. Eur J Endocrinol 141:169-79.

Besnard, N., C. Pisselet, D. Monniaux, A. Locatelli, F. Benne, F. Gasser, F. Hatey, and P. Monget. 1996. Expression of messenger ribonucleic acids of insulin-like growth factor binding proteins-2, -4 and -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. Biol. Reprod. 55: 1356-67.

Beukers, M. W., Y. Oh, H. Zhang, N. Ling, and R. G. Rosenfeld. 1991. [Leu27] insulin-like growth factor II is highly selective for the type-II IGF receptor in binding, cross-linking and thymidine incorporation experiments. Endocrinology 128:1201-3.

Boisclair, Y. R., Y. W. Yang, J. M. Stewart, and M. M. Rechler. 1994. Insulin-like growth factor-I and insulin stimulate the synthesis of IGF-binding protein-2 in a human embryonic kidney cell line. Growth Regul 4:136-46.

Bridges, T. S., T. R. Davidson, C. S. Chamberlain, R. D. Geisert, and L. J. Spicer. 2002. Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. J Anim Sci 80:179-90.

Brown, T. A. and T. D. Braden. 2001. Expression of insulin-like growth factor binding protein (IGFBP)-3 and the effects of IGFBP-2 and -3 in the bovine corpus luteum. Dom. Anim. Endo. 20: 203-16.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169-93.

Camacho-Hubner, C., W. H. Busby Jr, R. H. McCusker, G. Wright, and D. R. Clemmons. 1992. Identification of the forms of insulin-like growth factor-binding proteins produced by human fibroblasts and the mechanisms that regulate their secretion. J Biol Chem 267:11949-56.

Cataldo, N. A., T. K. Woodruff, and L. C. Giudice. 1993. Regulation of insulin-like growth factor binding protein production by human luteinizing granulosa cells cultured in defined medium. J Clin Endocrinol Metab 76:207-15.

Chamberlain, C. S. and L. J. Spicer. 2001. Hormonal control of ovarian cell production of insulin-like growth factor binding proteins. Mol Cell Endocrinol 182:69-81.

Cohick, W. S., A. Gockerman, and D. R. Clemmons. 1993. Vascular smooth muscle cells synthesize two forms of insulin-like growth factor binding proteins which are regulated differently by the insulin-like growth factors. J Cell Physiol 157:52-60.

Cohick, W. S., M. A. McGuire, D. R. Clemmons and D. E. Bauman. 1992. Regulation of insulin-like growth factor-binding proteins in serum and lymph of lactating cows by somatotropin. Endocrinology 130:1508-14.

Conover, C. A., G. F. Faessen, K. E. Ilg, Y. A. Chandrasekher, M. Christiansen, M. T. Overgaard, C. Oxvig, and L. C. Giudice. 2001. Pregnancy-associated plasma protein-A is the insulin-like growth factor binding protein-4 protease secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum. Endocrinology 142:2155.

Conover, C. A. and M. C. Kiefer. 1993. Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells. J Clin Endocrinol Metab 76:1153-9.

Conover, C. A., M. C. Kiefer, and J. Zapf. 1993. Posttranslational regulation of insulin-like growth factor binding protein-4 in normal and transformed human fibroblasts. Insulin-like growth factor dependence and biological studies. J Clin Invest 91:1129-37.

Dahlfors, G. and H. F. Arnqvist. 2000. Vascular endothelial growth factor and transforming growth factor-β1 regulate the expression of insulin-like growth factor-binding protein-3, -4 and -5 in large vessel endothelial cells. Endocrinology 141:2062-67.

Davidson, T. R., C. S. Chamberlain, T. S. Bridges, and L. J. Spicer. 2002. Effect of Follicle Size on In Vitro Production of Steroids and Insulin- Like Growth Factor (IGF)-I, IGF-II, and the IGF-Binding Proteins by Equine Ovarian Granulosa Cells. Biol Reprod 66:1640-8.

de la Sota, R. L., F. A. Simmen, T. Diaz, and W. W. Thatcher. 1996. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. Biol Reprod 55:803-12.

Dor, J., N. Costritsci, C. Pariente, J. Rabinovici, S. Mashiach, B. Lunenfeld, H. Kaneti, M. Seppala, R. Koistinen, and A. Karasik. 1992. Insulin-like growth factor-I and follicle-stimulating hormone suppress insulin-like growth factor binding protein-1 secretion by human granulosa-luteal cells. J Clin Endocrinol Metab 75:969-71.

Durham, S. K., A. Suwanichkul, A. O. Scheimann, D. Yee, J. G. Jackson, F. G. Barr, and D. R. Powell. 1999. FKHR binds the insulin response elementin the insulin-like growth factor binding protein-1 promoter. Endocrinology 140:3140-46.

Echternkamp, S. E., H. J. Howard, A. J. Roberts, J. Grizzle, and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biol Reprod 51:971-81.

Edwards, J. L., T. C. Hughey, A. B. Moore, and N. M. Cox. 1996. Depletion of insulin in streptozocin-induced-diabetic pigs alters estradiol, insulin-like growth factor (IGF)-I and IGF binding proteins in cultured ovarian follicles. Biol Reprod 55:775-81.

El-Roeiy, A., X. Chen, V. J. Roberts, S. Shimasaki, N. Ling, D. LeRoith, C. T. Roberts, Jr., and S. S. C. Yen. 1994. Expression of the genes encoding the insulin-like growth factors (IGF-I and -II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. J Clin. Endo. Metab. 78: 1488-96.

Erickson G.F., Danmei L., Shunichi S., Nicholas L., Weitsman S.R. and , and Magoffin D.A. 1995. Insulin-like growth factor-1 (IGF-1) stimulates IGF binding protein system in rat theca interstitial cells. Endocrine 3, 525-531.

Erickson, G. F., D. Li, R. Sadrkhanloo, X. J. Liu, S. Shimasaki, and N. Ling. 1994. Extrapituitary actions of gonadotropin-releasing hormone: stimulation of insulin-like growth factor-binding protein-4 and atresia. Endocrinology 134:1365-72.

Erickson, G. F., A. Nakatani, N. Ling, and S. Shimasaki. 1992. Cyclic changes in insulin -like growth factor-binding protein-4 messenger ribonucleic acid in the rat ovary. Endocrinology 130:625-36.

Erickson, G. F., A. Nakatani, N. Ling, and S. Shimasaki. 1992. Localization of insulin-like growth factor-binding protein-5 messenger ribonucleic acid in rat ovaries during the estrous cycle. Endocrinology 130:1867-78.

Erondu, N. E., B. Toland, M. Boes, B. Dake, D. R. Moser, and R. S. Bar. 1997. Bovine insulin-like growth factor binding protein-3: Organization of the chromosomal gene and functional analysis of its promoter. Endocrinology 138: 2856-62.

Fazleabas, A. T., R. C. Jaffe, H. G. Verhage, G. Waites, and S. C. Bell. 1989. An insulin-like growth factor-binding protein in the baboon (Papio anubis) endometrium: synthesis, immunocytochemical localization, and hormonal regulation. Endocrinology 124:2321-9.

Feldman, E. L. and A. E. Randolph. 1994. Regulation of insulin-like growth factor binding protein synthesis and secretion in human retinal pigment epithelial cells. J Cell Physiol 158:198-204.

Fielder, P. J., H. Pham, E. Y. Adashi, and R. G. Rosenfeld. 1993. Insulin-like growth factors (IGFs) block FSH-induced proteolysis of IGF- binding protein-5 (BP-5) in cultured rat granulosa cells. Endocrinology 133:415-8.

Foss, D. L., M. J. Baarsch, and M. P. Murtaugh. 1998. Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. Anim Biotechnol 9:67-78.

Fowler, D. J., K. H. Nicolaides, and J. P. Miell. 2000. Insulin-like growth factor binding protein-1 (IGFBP-1): a multifunctional role in the human female reproductive tract. Hum Reprod Update 6:495-504.

Fraser, H. M., S. F. Lunn, H. Kim, W. C. Duncan, F. E. Rodger, P. J. Illingworth, and G. F. Erickson. 2000. Changes in insulin-like growth factor-binding protein-3 messenger ribonucleic acid in endothelial cells of the human corpus luteum: a possible role in luteal development and rescue. J Clin Endocrinol Metab 85:1672-7.

Fraser, H. M., S. F. Lunn, H. Kim and G. F. Erickson. 1998. Insulin-like growth factor binding protein-3 mRNA expression in endothelial cells of the primate corpus luteum. Hum Reprod 13:2180-85.

Froment, P., D. Seurin, S. Hembert, J. E. Levine, C. Pisselet, D. Monniaux, M. Binoux and P. Monget. 2002. Reproductive abnormalities in human IGF binding protein-1 transgenic female mice. Endocrinology 143: 1801-1808.

Funston, R. N., G. E. Seidel Jr, J. Klindt, and A. J. Roberts. 1996. Insulin-like growth factor I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. Biol Reprod 55:1390-6.

Gabbitas, B. and E. Canalis. 1997. Growth factor regulation of insulin-like growth factor binding protein-6 expression in osteoblasts. J Cell Biochem 66:77-86.

Gersak, K., M. Seppala, and M. Us-Krasovec. 1999. The same luteinized granulosa-luteal cells from normal and superovulated human chorionic gonadotropin (hCG)-stimulated cycles contain hCG and insulin-like growth factor binding protein-1. Fert Steril 71: 902-6.

Ginther, O. J. 2000. Selection of the dominant follicle in cattle and horses. Anim Reprod Sci 60-61:61-79.

Ginther, O. J., M. C. Wiltbank, P. M. Fricke, J. R. Gibbons, and K. Kot. 1996. Selection of the dominant follicle in cattle. Biol Reprod 55:1187-94.

Giudice, L. C., A. A. Milki, D. A. Milkowski, and I. el Danasouri. 1991. Human granulosa contain messenger ribonucleic acids encoding insulin-like growth factor-binding proteins (IGFBPs) and secrete IGFBPs in culture. Fertil Steril 56:475-80.

Greisen, S., A. Flyvbjerg, T. Ledet, and P. Ovesen. 2002. Regulation of insulin-like growth factor binding protein secretion by human granulosa luteal cells in a polycystic ovary-like environment. Fertil Steril 78:162-8.

Grimes, R. W., J. A. Barber, S. Shimasaki, N. Ling, and J. M. Hammond. 1994. Porcine ovarian granulosa cells secrete insulin-like growth factor- binding proteins-4 and -5 and express their messenger ribonucleic acids: regulation by follicle-stimulating hormone and insulin-like growth factor-1. Biol Reprod 50:695-701.

Grimes, R. W. and J. M. Hammond. 1992. Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. Endocrinology 131:553-8.

Grimes, R. W., S. E. Samaras, J. A. Barber, S. Shimasaki, N. Ling, and J. M. Hammond. 1992. Gonadotropin and cAMP modulation of IGF binding protein production in ovarian granulosa cells. Am J Physiol 262:E497-503.

Gruaz, N. M., V. d'Alleves, Y. Charnay, A. Skottner, S. Ekvarn, L. Fryklund, and M. L. Aubert. 1997. Effects of constant infusion with insulin-like growth factor-I (IGF-I) to immature female rats on body weight gain, tissue growth and sexual function. Evidence that such treatment does not affect sexual maturation of fertility. Endocrine 6:11-9.

Guo, Y. S., C. M. Townsend Jr, G. F. Jin, R. D. Beauchamp, and J. C. Thompson. 1995. Differential regulation by TGF-beta 1 and insulin of insulin-like growth factor binding protein-2 in IEC-6 cells. Am J Physiol 268:E1199-204.

Hill, D. J., C. Camacho-Hubner, P. Rashid, A. J. Strain, and D. R. Clemmons. 1989. Insulin-like growth factor (IGF)-binding protein release by human fetal fibroblasts: dependency on cell density and IGF peptides. J Endocrinol 122:87-98.

Holst, N., K. H. Kierulf, M. Seppala, R. Koistinen, and M. B. Jacobsen. 1997. Regulation of insulin-like growth factor-binding protein-1 and progesterone secretion from human granulosa-luteal cells: effects of octreotide and insulin. Fertil Steril 68:478-82.

Howard, H. J. and J. J. Ford. 1992. Relationships among concentrations of steroids, inhibin, insulin-like growth factor-1 (IGF-1), and IGF-binding proteins during follicular development in weaned sows. Biol Reprod 47:193-201.

Huynh, H. 2000. Post-transcriptional and post-translational regulation of insulin-like growth factor binding protein-3 and -4 by insulin-like growth factor-I in uterine myometrial cells. Growth Horm IGF Res 10:20-7.

Ingman, W. V., P. C. Owens, and D. T. Armstrong. 2000. Differential regulation by FSH and IGF-I of extracellular matrix IGFBP- 5 in bovine granulosa cells: effect of association with the oocyte. Mol Cell Endocrinol 164:53-8.

Irwin, J. C., B. A. Dsupin, and L. C. Giudice. 1995. Regulation of insulin-like growth factor-binding protein-4 in human endometrial stromal cell cultures: evidence for ligand-induced proteolysis. J Clin Endocrinol Metab 80:619-26.

Irwin, J. C., L. F. Suen, G. H. Faessen, R. M. Popovici, and L. C. Giudice. 2001. Insulin-like growth factor (IGF)-II inhibition of endometrial stromal cell tissue inhibitor of metalloproteinase-3 and IGF-binding protein-1 suggests paracrine interactions at the decidua:trophoblast interface during human implantation. J Clin Endocrinol Metab 86:2060-4.

Iwashita, M., Y. Kudo, and Y. Takeda. 1998. Effect of follicle stimulating hormone and insulin-like growth factors on proteolysis of insulin-like growth factor binding protein-4 in human granulosa cells. Mol Hum Reprod 4: 401-405.

Jalkanen, J., A. M. Suikkari, R. Koistinen, R. Butzow, O. Ritvos, M. Seppala, and T. Ranta. 1989. Regulation of insulin-like growth factor-binding protein-1 production in human granulosa-luteal cells. J Clin Endocrinol Metab 69:1174-9.

Jones, J. I. and D. R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34.

Kassem, M., R. Okazaki, D. De Leon, S. A. Harris, J. A. Robinson, T. C. Spelsberg, C. A. Conover, and B. L. Riggs. 1996. Potential mechanism of estrogen-mediated decrease in bone formation: estrogen increases production of inhibitory insulin-like growth factor-binding protein-4. Proc Assoc Am Physicians 108:155-64.

Kim, I., A. Manni, J. Lynch, and J. M. Hammond. 1991. Identification and regulation of insulin-like growth factor binding proteins produced by hormone-dependent and -independent human breast cancer cell lines. Mol Cell Endocrinol 78:71-8.

Laursen, L. S., M. T. Overgaard, R. Soe, H. B. Boldt, L. Sottrup-Jensen, L. C. Giudice, C. A. Conover, and C. Oxvig. 2001. Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. FEBS Letters 504: 36-40.

Liu, H. C., Z. Y. He, C. Mele, M. Damario, O. Davis, and Z. Rosenwaks. 1997. Hormonal regulation of expression of messenger RNA encoding insulin-like growth factor binding proteins in human endometrial stromal cells cultured in vitro. Mol Hum Reprod 3:21-6.

Liu, J., A. T. Koenigsfeld, T. C. Cantley, C. K. Boyd, Y. Kobayashi, and M. C. Lucy. 2000. Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual components of the ovarian insulin-like growth factor system. Biol Reprod 63:942-52.

Liu, X. J. and N. Ling. 1993. Regulation of IGFBP-4 and -5 expression in rat granulosa cells. Adv Exp Med Biol 343:367-76.

Liu, X. J., M. Malkowski, Y. Guo, G. F. Erickson, S. Shimasaki, and N. Ling. 1993. Development of specific antibodies to rat insulin-like growth factor- binding proteins (IGFBP-2 to -6): analysis of IGFBP production by rat granulosa cells. Endocrinology 132:1176-83.

Martikainen, H., R. Koistinen, and M. Seppala. 1992. The effect of estrogen level on glucose-induced changes in serum insulin-like growth factor binding protein-1 concentration. Fertil Steril 58:543-6.

Martin, J. L., J. A. Coverley, S. T. Pattison, and R. C. Baxter. 1995. Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. Endocrinology 136:1219-26.

Mason, H. D., R. Margara, R. M. Winston, M. Seppala, R. Koistinen, and S. Franks. 1993. Insulin-like growth factor-I (IGF-I) inhibits production of IGF-binding protein-1 while stimulating estradiol secretion in granulosa cells from normal and polycystic human ovaries. J Clin Endocrinol Metab 76:1275-9.

May, J. V., J. P. Frost, and D. W. Schomberg. 1988. Differential effects of epidermal growth factor, somatomedin-C/insulin- like growth factor I, and transforming growth factor-beta on porcine granulosa cell deoxyribonucleic acid synthesis and cell proliferation. Endocrinology 123:168-79.

Mazerbourg, S., M. T. Overgaard, C. Oxvig, M. Christiansen, C. A. Conover, I. Laurendeau, M. Vidaud, G. Tosser-Klopp, J. Zapf, and P. Monget. 2001. Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. Endocrinology 142:5243-53.

Mazerbourg, S., J. Zapf, R. S. Bar, D. R. Brigstock, and P. Monget. 2000. Insulin-like growth factor (IGF)-binding protein-4 proteolytic degradation in bovine, equine, and porcine preovulatory follicles: regulation by IGFs and heparin-binding domain-containing peptides. Biol Reprod 63:390-400.

Mihm, M., E. J. Austin, T. E. Good, J. L. Ireland, P. G. Knight, J. F. Roche, and J. J. Ireland. 2000. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. Biol Reprod 63:811-9.

Molnar, P. and L. J. Murphy. 1994. Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. J Mol Endocrinol 13:59-67.

Mondschein, J. S., S. A. Smith, and J. M. Hammond. 1990. Production of insulin-like growth factor binding proteins (IGFBPs) by porcine granulosa cells: identification of IGFBP-2 and -3 and regulation by hormones and growth factors. Endocrinology 127:2298-306.

Monniaux, D. and C. Pisselet. 1992. Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro. Biol Reprod 46:109-19.

Nakatani, A., S. Shimasaki, G. F. Erickson, and N. Ling. 1991. Tissue-specific expression of four insulin-like growth factor-binding proteins (1, 2, 3, and 4) in the rat ovary. Endocrinology 129: 1521-29.

National Agricultural Statistical Service (NASS). 2002. Cattle Inventory for United States. July, 19: 1-7.

Nicholas, B., R. K. Scougall, D. G. Armstrong and R. Webb. 2002. Changes in insulin-like growth factor binding proteins during bovine follicular development. Reproduction 124: 439-46.

Orlowski, C. C., G. T. Ooi, D. R. Brown, Y. W. Yang, L. Y. Tseng, and M. M. Rechler. 1991. Insulin rapidly inhibits insulin-like growth factor-binding protein-1 gene expression in H4-II-E rat hepatoma cells. Mol Endocrinol 5:1180-7.

Overgaard, M. T., H. B. Boldt, L. S. Laursen, L. Sottrup-Jensen, C. A. Conover, and C. Oxvig. 2001. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. J Biol Chem 276: 21849-53.

Ovesen, P., H. J. Ingerslev, H. Orskov, and T. Ledet. 1994. Effect of growth hormone on steroidogenesis, insulin-like growth factor- I (IGF-I) and IGF-binding protein-1 production and DNA synthesis in cultured human luteinized granulosa cells. J Endocrinol 140:313-9.

Poretsky, L., B. Chun, H. C. Liu, and Z. Rosenwaks. 1996. Insulin-like growth factor II (IGF-II) inhibits insulin-like growth factor binding protein I (IGFBP-1) production in luteinized human granulosa cells with a potency similar to insulin-like growth factor I (IGF-I) and higher than insulin. J Clin Endocrinol Metab 81:3412-4.

Poretsky, L., D. Seto-Young, A. Shrestha, S. Dhillon, M. Mirjany, H. C. Liu, M. C. Yih, and Z. Rosenwaks. 2001. Phosphatidyl-inositol-3 kinase-independent insulin action pathway(s) in the human ovary. J Clin Endocrinol Metab 86:3115-9.

Powell, D. R., A. Suwanichkul, M. L. Cubbage, L. A. DePaolis, M. B. Snuggs, and P. D. Lee. 1991. Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1. J Biol Chem 266:18868-76.

Putowski, L., R. M. Rohan, D. S. Choi, W. J. Scherzer, E. Ricciarelli, J. Mordacq, K. E. Mayo, and E. Y. Adashi. 1997. Rat ovarian insulin-like growth factor binding protein-4: a hormone- dependent granulosa cell-derived antigonadotropin. J Soc Gynecol Invest 4:144-51.

Putowski, L. R., D. Choi, J. Mordacq, W. J. Scherzer, K. E. Mayo, E. Y. Adashi, and R. M. Rohan. 1995. In vivo hormonal regulation of insulin-like growth factor binding protein-5 mRNA in the immature rat ovary. J Soc Gynecol Invest 6: 735-42.

Qin, C., P. Singh, and S. Safe. 1999. Transcriptional activation of insulin-like growth factor-binding protein-4 by 17β-estradiol in MCF-7 cells: Role of estrogen receptor-Sp1 complexes. Endocrinology 140: 2501-8.

Rajaram, S., D. J. Baylink, and S. Mohan. 1997. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18:801-31.

Rehault, S., P. Monget, S. Mazerbourg, R. Tremblay, N. Gutman, F. Gauthier, and T. Moreau. 2001. Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur J Biochem 268: 2960-68.

Rhodes, F. M., A. J. Peterson and P. D. Jolly. 2001. Gonadotrophin responsiveness, aromatase activity and insulin-like growth factor binding protein content of bovine ovarian follicles during the first follicular wave. Reproduction 122: 561-69.

Ricciarelli, E., E. R. Hernandez, A. Hurwitz, E. Kokia, R. G. Rosenfeld, J. Schwander, and E. Y. Adashi. 1991. The ovarian expression of the antigonadotropic insulin-like growth factor binding protein-2 is theca-interstitial cell-selective: evidence for hormonal regulation. Endocrinology 129:2266-8.

Ricciarelli, E., E. R. Hernandez, C. Tedeschi, L. F. Botero, E. Kokia, R. M. Rohan, R. G. Rosenfeld, A. L. Albiston, A. C. Herington, and E. Y. Adashi. 1992. Rat ovarian insulin-like growth factor binding protein-3: a growth hormone-dependent theca-interstitial cell-derived antigonadotropin. Endocrinology 130:3092-4.

Rinderknecht, E. and R. E. Humbel. 1978a. The amino acid sequence of human insulin -like growth factor I and its structural homology with proinsulin. J Biol Chem 253:2769-76.

Rinderknecht, E. and R. E. Humbel. 1978b. Primary structure of human insulin-like growth factor II. FEBS Lett 89:283-6.

Ripa, P., I Robertson, D. Cowley, M. Harris, I. B. Masters, and A. M. Cotterill. 2002. The relationship between insulin secretion, the insulin-like growth factor axis in children with cystic fibrosis. Clin Endocrinol 56: 383-89.

Rivera, G. M. and J. E. Fortune. 2001. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. Biol Reprod 65:112-8.

Rohan, R. M., E. Ricciarelli, M. C. Kiefer, C. E. Resnick, and E. Y. Adashi. 1993. Rat ovarian insulin-like growth factor-binding protein-6: a hormonally regulated theca-interstitial-selective species with limited antigonadotropic activity. Endocrinology 132:2507-12.

Rosato, R., K. Gerland, H. Jammes, N. Bataille-Simoneau, B. Segovia, L. Mercier, and A. Groyer. 2001. The IGFBP-3 mRNA and protein levels are IGF-I-dependent and GH- independent in MG-63 human osteosarcoma cells. Mol Cell Endocrinol 175:15-27.

Samaras, S. E., H. D. Guthrie, J. A. Barber and J. M. Hammond. 1993. Expression of the mRNAs for the insulin-like growth factors and their binding proteins during the development of the porcine ovarian follicle. Endocrinology 133: 2395-98.

Samaras, S. E., D. R. Hagen, S. Shimasaki, N. Ling, and J. M. Hammond. 1992. Expression of insulin-like factor-binding protein-2 and -3 messenger ribonucleic acid in the porcine ovary: localization and physiological changes. Endocrinology 130: 2739- 44.

San Roman, G. A. and D. A. Magoffin. 1993. Insulin-like growth factor-binding proteins in healthy and atretic follicles during natural menstrual cycles. J Clin Endocrinol Metab 76:625-32.

San Roman, G. A. and D. A. Magoffin. 1992. Insulin-like growth factor binding proteins in ovarian follicles from women with polycystic ovarian disease: cellular source and levels in follicular fluid. J Clin Endocrinol Metab 75:1010-6.

Sayre, B. L., R. Taft, E. K. Inskeep and J. Killefer. 2000. Increased expression of insulin-like growth factor binding protein-1 during induced regression of bovine corpora lutea. Biol Reprod 63: 21-29.

Schams, D., B. Berisha, M. Kosmann, and W. M. Amselgruber. 2002. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. Domest Anim Endocrinol 22:51-72.

Schams, D., B. Berisha, M. Kosmann, R. Einspanier, and W. M. Amselgruber. 1999. Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. Domest Anim Endocrinol 17:279-85.

Scharf, J. G., W. Schmidt-Sandte, S. A. Pahernik, H. G. Koebe, and H. Hartmann. 1996. Synthesis of insulin-like growth factor binding proteins and of the acid-labile subunit of the insulin-like growth factor ternary binding protein complex in primary cultures of human hepatocytes. Hepatology 23:424-30.

Schmid, C., M. Ernst, J. Zapf, and E. R. Froesch. 1989. Release of insulin-like growth factor carrier proteins by osteoblasts: stimulation by estradiol and growth hormone. Biochem Biophys Res Commun 160:788-94.

Schmid, C., I. Schlapfer, M. Waldvogel, P. J. Meier, J. Schwander, M. Boni-Schnetzler, J. Zapf, and E. R. Froesch. 1992. Differential regulation of insulin-like growth factor binding protein (IGFBP)-2 mRNA in liver and bone cells by insulin and retinoic acid in vitro. FEBS Lett 303:205-9.

Schmittgen, T. D. and B. A. Zakrajsek. 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69-81.

Shimasaki, S., F. Uchiyama, M. Shimonaka, and N. Ling. 1990. Molecular cloning of the cDNAs encoding a novel insulin-like growth factor-binding protein from rat and human. Mol Endocrinol 4:1451-8.

Silva, J. M. and C. A. Price. 2002. Insulin and IGF-I are necessary for FSH-induced cytochrome P450 aromatase but not cytochrome P450 side-chain cleavage gene expression in oestrogenic bovine granulosa cell in vitro. J Endocrinology 174: 499-507.

Simmons, J. G., E. C. Hoyt, J. K. Westwick, D. A. Brenner, J. B. Pucilowska and P. K. Lund. 1995. Insulin-like growth factor-I and epidermal growth factor interact to regulate growth and gene expression in IEC-6 intestinal epithelial cells. Mol Endo 9: 1157-65.

Simpson, R. B., C. C. Chase Jr, L. J. Spicer, J. A. Carroll, A. C. Hammond, and T. H. Welsh Jr. 1997. Effect of exogenous estradiol on plasma concentrations of somatotropin, insulin-like growth factor-I, insulin-like growth factor binding protein activity, and metabolites in ovariectomized Angus and Brahman cows. Domest Anim Endocrinol 14:367-80.

Simpson, R. B., C. C. Chase Jr, L. J. Spicer, R. K. Vernon, A. C. Hammond, and D. O. Rae. 1994. Effect of exogenous insulin on plasma and follicular insulin-like growth factor I, insulin-like growth factor binding protein activity, follicular oestradiol and progesterone, and follicular growth in superovulated Angus and Brahman cows. J Reprod Fertil 102:483-92.

Spicer, L. J., E. Alpizar, and S. E. Echternkamp. 1993. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. J Anim Sci 71:1232-41.

Spicer, L. J. and C. S. Chamberlain. 2002. Estradiol and luteinizing hormone regulation of insulin-like growth factor binding protein production by bovine granulosa and thecal cells. Endocrine 17:161-8.

Spicer, L. J. and C. S. Chamberlain. 1999. Insulin-like growth factor binding protein-3: its biological effect on bovine granulosa cells. Domest Anim Endocrinol 16:19-29.

Spicer, L. J., C. S. Chamberlain, and S. M. Maciel. 2002. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest Anim Endocrinol 22:237-54.

Spicer, L. J., C. S. Chamberlain, and G. L. Morgan. 2001. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. Domest Anim Endocrinol 21:1-15.

Spicer, L. J. and S. E. Echternkamp. 1986. Ovarian follicular growth, function and turnover in cattle: a review. J Anim Sci 62:428-51.

Spicer, L. J. and S. E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest Anim Endocrinol 12:223-45.

Spicer, L. J., R. E. Stewart, P. Alvarez, C. C. Francisco, and B. E. Keefer. 1997. Insulin-like growth factor-binding protein-2 and -3: their biological effects in bovine thecal cells. Biol Reprod 56:1458-65.

Stevenson, J.S. 2000. Hoard's Dairyman (January 15): 22.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, and B. E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 73:3719-31.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E. Echternkamp. 1996. 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. Endocrinology 137:2842-50.

Sunic, D., J. D. McNeil, D. L. Andress, and D. A. Belford. 1998. Insulin-like growth factor binding protein-5 proteolytic activity in ovine articular chondrocyte culture. Biochem Biophys Acta 1425: 567-76.

Suzuki, T., P. J. Higgins, and D. R. Crawford. 2000. Control selection for RNA quantitation. Biotechniques 29:332-7.

Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen. 1999. Housekeeping genes as internal standards: use and limits. J Biotechnol 75:291-5.

Thrailkill, K. M., D. R. Clemmons, W. H. Busby Jr. and S. H. 1990 Differential Regulation of Insulin-like Growth Factor Binding Protein Secretion from Human Decidual Cells by IGF-I, Insulin, and Relaxin. J. Clin. Invest 86, 878-883.

Tomizawa, M., A. Kumar, V. Perrot, J. Nakae, D. Accili, and M. M. Rechler. 2000. Insulin inhibits the activation of transcription by a C-terminal fragment of the forkhead transcription factor FKHR. J Biol Chem 275: 7289-95.

Voge, J. L., D. T. Allen, J. R. Malayer and L. J. Spicer. 2001. Expression of insulin-like growth factor-binding protein-2, -3, -4, and -5 in fresh versus cultured bovine granulosa and theca cells. Journal of Animal Science 79 (Suppl. 1): 33.

Voutilainen, R., S. Franks, H. D. Mason, and H. Martikainen. 1996. Expression of insulin-like growth factor (IGF), IGF-binding protein, and IGF receptor messenger ribonucleic acids in normal and polycystic ovaries. J Clin Endocrinol Metab 81:1003-8.

Wandji, S. A., J. E. Gadsby, F. A. Simmen, J. A. Barber and J. M. Hammond. 2000. Porcine ovarian cells express messenger ribonucleic acids for the acid-labile subunit and insulin-like growth factor binding protein-3 during follicular and luteal phases of the estrous cycle. Endocrinology 141: 2638-47.

Wandji, S. A., T. L. Wood, J. Crawford, S. W. Levison, and J. M. Hammond. 1998. Expression of mouse ovarian insulin growth factor system components during follicular development and atresia. Endocrinology 139:5205-14.

Yuan, W., B. Bao, H. A. Garverick, R. S. Youngquist, and M. C. Lucy. 1998. Follicular dominance in cattle is associated with divergent patterns of ovarian gene

expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. Dom Anim Endocrinol 15: 55-63.

Zhou, J., O. O. Adesanya, G. Vatzias, J. M. Hammond and C. A. Bondy. 1996. Selective expression of insulin-like growth factor system components during porcine ovary follicular selection. Endocrinology 137: 4893-901.

CHAPTER III

HORMONAL CONTROL OF INSULIN-LIKE GROWTH FACTOR BINDING-PROTEIN mRNA EXPRESSION IN BOVINE GRANULOSA AND THECA CELLS: QUANTIFICATION BY REAL-TIME RT-PCR

ABSTRACT

To determine if expression levels of insulin-like growth factor binding proteins (IGFBP)-2, -3 and -4 mRNA in bovine granulosa and theca cells are affected by various hormone treatments, granulosa and theca cells were collected from bovine ovarian follicles, cultured for 2 days in medium containing 10% FCS, and then treated for 24 h in serum-free medium with various hormones in a total of seven experiments. Amounts of IGFBP-2, -3 and -4 were quantitated using fluorescent quantitative real-time RT-PCR. Relative quantitation was done using the comparative cycle threshold (Ct) and all values are expressed as fold gene expression ($2^{-\Delta\Delta Ct}$) relative to the sample exhibiting the highest ΔCt within a particular IGFBP and experiment. Neither IGF-1 nor IGF-2 had an effect ($P > 0.10$) on IGFBP-2, -3 or -4 mRNA levels in small-follicle (1-5 mm) granulosa cells, whereas follicle-stimulating hormone (FSH) in the presence or absence of insulin increased ($P < 0.05$) IGFBP-3 mRNA but did not change IGFBP-2 or -4 mRNA levels. Luteinizing hormone (LH) in the absence of insulin also increased ($P < 0.05$) small-follicle granulosa cell IGFBP-3 mRNA levels but had no effect on IGFBP-3 mRNA levels in the presence of insulin. Estradiol was without effect on IGFBP-2, -3 or -4 mRNA levels in granulosa cells from small-follicles. In large-follicle (> 7.9 mm)

granulosa cells, 100 ng/ml of IGF-1 increased ($P < 0.05$) IGFBP-2 mRNA levels, while 100 ng/ml of IGF-2 had no effect ($P > 0.10$) on IGFBP-2, -3 and -4 mRNA levels. Also in large-follicle granulosa cells, insulin alone increased ($P < 0.05$) IGFBP-2 gene expression while LH, FSH and estradiol treatments were without any significant effect. In theca cells, IGF-2 decreased ($P < 0.05$) IGFBP-2 mRNA levels, but had no effect on IGFBP-3 or -4 mRNA expression. IGF-1 did not affect ($P > 0.10$) theca cell IGFBP-2, -3 or -4 mRNA levels. Insulin decreased ($P < 0.05$) IGFBP-4 expression by theca cells, but had no effect ($P > 0.10$) on thecal IGFBP-2 or -3 mRNA levels. Similarly, estradiol decreased ($P < 0.05$) thecal IGFBP-3 and -4 mRNA but not IGFBP-2 mRNA levels. These results suggest that expression of IGFBP-2, -3 and -4 mRNA by granulosa and theca cells are differentially regulated by various hormonal stimuli, therefore possibly modulating the amount of bioavailable IGFs to these cells.

INTRODUCTION

A normal 21-day estrous cycle in cattle is associated with a milieu of hormonal events, consisting of both peptide and steroid hormones, as well as growth factors, interacting to regulate ovarian follicular growth and function. Among these are insulin-like growth factor-1 (IGF-1) and IGF-2 which stimulate mitogenesis and steroidogenesis of granulosa and theca cells through autocrine, paracrine and endocrine mechanisms (for rev. see, Spicer and Echternkamp 1995; Adashi, 1998). In cattle, intrafollicular levels of IGF-1 and insulin-like growth factor binding protein-3 (IGFBP-3) remain relatively constant during follicular growth, while levels of IGFBP-2, -4 and -5 in follicular fluid fluctuate dramatically (de al Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996), indicating that the amount of bioavailable IGF-1 to the follicle is ultimately dictated by

changes in the levels of IGFBPs. Specifically, IGFBP-2, -4 and -5 are nearly absent in dominant nonovulatory (de la Sota et al., 1996; Stewart et al., 1996) and ovulatory (Funston et al., 1996; Spicer et al., 2001) follicles, with high concentrations detected in large subordinate and small follicles. Also as the nonovulatory dominant follicle is undergoing atresia (d 10 of estrous cycle) levels of IGFBP-2, -4 and -5 increase (Stewart et al., 1996).

These changes seen in intrafollicular levels of IGFBPs are likely attributed to hormone-induced changes in gene transcription and subsequent IGFBP production (Rajaram et al., 1997). For example, in cultured rat granulosa cells, follicle-stimulating hormone (FSH) decreases IGFBP-4 protein production as well as expression of its mRNA (Liu et al., 1993; Erickson et al., 1994). In cultured porcine granulosa cells, FSH inhibits and insulin stimulates IGFBP-2 production (Mondschein et al., 1990; Grimes et al., 1992). However, in bovine granulosa cells FSH was without effect, while insulin inhibited IGFBP-2/-5 production (Chamberlain and Spicer, 2001). Production of IGFBP-4 by ovine thecal cells was stimulated by luteinizing hormone (LH) (Armstrong et al., 1996), while no effect of LH was seen on bovine thecal cell IGFBP-4 production (Spicer and Chamberlain, 2002). Collectively, these studies indicate that IGFBP production by granulosa and thecal cells is hormonally regulated but the specific effect of a given hormone is likely species dependent. However, which specific IGFBP gene expression in bovine granulosa or theca cells is influenced by estradiol, IGF-1 or -2, or leptin has not been elucidated.

In addition to changes in IGFBP production, differences in total IGFBP proteins in follicular fluid and culture medium may in part be attributed to differences in protease

activity (Rajaram et al., 1997; Fortune et al., 2001; Spicer et al., 2001). In cattle, follicular fluid from dominant follicles was able to cleave IGFBP-4 and IGFBP-5 and this proteolytic activity was associated positively with levels of estradiol (Spicer et al., 2001). Similarly, rat granulosa cell conditioned medium cleaved radiolabelled IGFBP-5 into two smaller molecular weight bands (Fielder et al., 1993). Moreover, western immuno and ligand blotting techniques can cause confusion as to which specific IGFBP's production is being affected, due to lack of specificity of these techniques. For example previous work in bovine granulosa cells was not able to dissertain a large band on western ligand blotting because it comigrated with IGFBP-2/-5 (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002). Thus, in some situations it is necessary to measure changes in IGFBP mRNA levels to determine which specific IGFBP's production may be changing due to a specific treatment. The aim of this study was to determine the hormonal regulation of IGFBP mRNA levels in cultured bovine granulosa and theca cells using fluorescent real-time quantitative RT-PCR, with specific focus on the effects of estradiol, insulin, LH, FSH, leptin and IGFs.

MATERIALS AND METHODS

CELL CULTURE

The following reagents and hormones were used for cell culture: Dulbecco modified Eagle medium (DMEM), Ham's F-12, sodium bicarbonate, gentamicin, insulin (bovine; 28 U/mg), trypan blue, fetal calf serum (FCS), estradiol, protease, collagenase, hyaluronidase, and Dnase all obtained from Sigma Chemical Company (St. Louis, MO); ovine FSH (F1913; FSH activity, 15 X NIH-FSH-S1 U/mg) and bovine LH (L1914; LH activity 2.0 X NIH-LH-S1 U/mg) from Scripps Laboratories (San Diego, CA);

recombinant mouse leptin (Pepro Tech Inc., Rocky Hill, NJ); and recombinant human IGF-1 and IGF-2 from R & D Systems (Minneapolis, MN).

Ovaries from pregnant and nonpregnant dairy and beef cattle were obtained at a local abattoir, placed in saline (0.15 M NaCl) and brought back to the laboratory (<120 min). Granulosa cells from small (1-5 mm) and large (> 7.9 mm) follicles were collected by aspiration using a 20-gauge needle (38.1 mm diameter) and a 3 ml syringe as previously described (Langhout et al., 1991). Briefly, granulosa cells were washed two times in serum-free medium by centrifugation at 200 x g for 5 min, and then resuspended in serum-free media containing 1.25 mg/ml collagenase and 0.5 mg/ml Dnase to prevent clumping.

Theca cells were collected from large follicles (> 7.9 mm) by dissection as previously described (Stewart et al., 1995). After removal of granulosa cells by aspiration and scraping, the theca interna was torn into small pieces and digested for 1 h at 37° C on a rocking platform. Tissue that was not completely digested after enzymatic digestion was removed via filtration through a sterile syringe filter holder with a metal screen (149 μm mesh; Gelman, Ann Arbor, MI). The theca cells were then washed in serum-free medium by centrifugation at 50 x g for 5 min. Theca cells were then resuspended in serum-free media containing of 1.25 mg/ml collagenase and 0.5 mg/ml Dnase to prevent clumping. The percentage of viable granulosa cells from small and large follicles and theca cells from large follicles was determined by trypan blue exclusion method and averaged: 40.6 ± 17.7, 74.0 ± 10.7, and 94.2 ± 4.2%, respectively.

Cells were then cultured at 38° C with 95% air 5% CO_2 in medium consisting of a 1:1 mixture of DMEM and Ham's F-12 containing 0.12 mM gentamicin, 2.0 mM

glutamine, and 38.5 mM sodium bicarbonate. Approximately 2.0×10^5 viable cells were placed into each well of 24-well Falcon multiwell plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) containing 1 ml medium. During the first 48 h of incubation, cells were cultured in medium containing 10% FCS with a medium change at 24 h. At the conclusion of the first 48 h of incubation, cells were then washed twice with 0.5 ml serum-free medium and hormonal treatments were applied for 24 h. Media was then aspirated from each well and 0.5 ml of Trizol reagent (Life Technologies, Inc., Gaithersburg MD) was placed into one well to lyse cells and then transferred into one replicate well such that cells from four wells from each treatment within an experiment were combined to form two replicate samples.

EXPERIMENTAL DESIGN

Experiment 1 was designed to determine the effects estradiol, FSH, and LH on basal IGFBP gene expression in granulosa cells collected from small bovine follicles. To accomplish this, cells were cultured in medium containing 10% FCS for 48 h and washed twice with 0.5 ml of serum-free medium as described earlier. Cells were then exposed for 24 h to one of the following hormonal treatments: Control (no additions), estradiol (300 ng/ml), FSH (30 ng/ml), LH (30 ng/ml), FSH (30 ng/ml) + estradiol (300 ng/ml), and LH (30 ng/ml) + estradiol (300 ng/ml). After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

In Experiment 2, small-follicle granulosa cells were cultured in order to determine the effect of estradiol, FSH and (or) LH in the presence of insulin on IGFBP gene expression. Granulosa cells were cultured as described in Experiment 1 except that during the last 24 h of culture cells were treated with one of the following six treatments:

insulin (100 ng/ml, to all wells unless otherwise stated), insulin + low FSH (3 ng/ml), insulin + high FSH (30 ng/ml), insulin + LH (30 ng/ml), insulin + high FSH (30 ng/ml) + low estradiol (3 ng/ml), and insulin + high FSH (30 ng/ml) + high estradiol (300 ng/ml). After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

Experiment 3 was carried out to compare the effects of IGF-1 and -2 on IGFBP gene expression in small-follicle granulosa cells. Granulosa cells were cultured as described in Experiment 1 except that during the last 24 h of culture cells were treated with media containing no additions (Control), 100 ng/ml of IGF-1 or 100 ng/ml of IGF-2. After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

Experiment 4 was designed to test the effects of insulin, FSH, LH, and estradiol on expression IGFBP mRNAs in granulosa cells collected from large follicles (> 7.9 mm). Large-follicle granulosa cells were cultured as described in Experiment 1 except that during the last 24 h of culture, cells were treated with: Control (no additions), insulin (100 ng/ml, all other wells containing insulin at same concentration), insulin + FSH (30 ng/ml), insulin + LH (30 ng/ml), insulin + FSH + low estradiol (3 ng/ml), and insulin + FSH + high estradiol (300 ng/ml). After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

Experiment 5 was designed similar to Experiment 3 to test the effects of IGF-1 and -2 as well as leptin and low doses of FSH and IGF-1 on moderately differentiated large-follicle granulosa cells. Granulosa cells were cultured as described in Experiment 1 except that during the last 24 h of culture cells were treated with: Control (no additions),

IGF-1 (100 ng/ml), IGF-2 (100 ng/ml), IGF-1 (3 ng/ml), FSH (3 ng/ml) or leptin (100 ng/ml). After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

Experiment 6 was carried out to determine the effect of insulin, estradiol, and LH on IGFBP gene expression in theca cells. Large-follicle theca cells were cultured in medium containing 10% FCS for 48 h and washed twice with 0.5 ml of serum-free medium as described earlier. Cells were then treated for 24 h with one of the following six treatments: Control (no additions), LH (30 ng/ml), estradiol (500 ng/ml), insulin (100 ng/ml), LH + insulin, and estradiol + insulin. After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

Experiment 7 was carried out to compare the effects of IGF-1 and -2 on the expression of IGFBPs in theca cells of large follicles. Theca cells were cultured as described in Experiment 6 except that during the last 24 h of culture, cells were treated with: Control (no additions), 100 ng/ml of IGF-1 or 100 ng/ml of IGF-2. After 24 h of treatment, media was aspirated and cells lysed for RNA extraction as described earlier.

mRNA ANALYSES

RNA extraction. Total cellular RNA was isolated from cultured granulosa and theca cells by lysing them in 0.50 ml of Trizol reagent (Life Technologies, Inc., Gaithersurg, MD). Lysed cells were transferred to 1.5 ml eppendorf tubes and then incubated in Trizol for 5 min at 22° C. Next, 0.10 ml of chloroform (Sigma Chemical Co., St. Louis, MO) was added and each sample vortexed for 15 sec. Following a 3 min incubation at 22° C, samples were centrifuged (3500 x g) for 30 min at 4° C. The upper aqueous phase was transferred to a new eppendorf tube and RNA precipitated with 0.250

ml of isopropyl alcohol (Pierce Chemical Company, Rockford, IL). Samples were gently mixed by hand and then incubated at 22° C for 10 min, followed by centrifugation at 3500 x g for 10 min at 4° C. The supernatant was then removed and the RNA pellet washed with 0.50 ml of 75% ethanol and then centrifuged as before for 5 min. Ethanol supernatant was removed and RNA pellet allowed to dry for 5 min at 22° C. The RNA was then dissolved in 0.030 ml of T.E. buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.4). Quantification of total RNA was determined spectrophotometrically at the 260 nm reading after which samples were aliquoted and stored at -80° C until used for quantification of IGFBP-2, -3 and -4 mRNA expression. Just prior to use, a RNA aliquot was thawed on ice for 3-5 min.

Quantitative RT-PCR. Fluorescent real-time quantitative RT-PCR was used to determine differences in mRNA expression levels for IGFBP-2, -3 and -4 between treatments within bovine granulosa and theca cells in each experiment. Expression levels were quantitated using one-step RT-PCR reaction following manufacture's specifications with modifications for Taqman® Gold RT-PCR kit (P/N N808-0233; PE Biosystems, Foster City, CA). The Taqman probes for each IGFBP contain a 5' reporter dye (TET) and 3' quencher dye (TAMRA). Cleavage of this probe after annealing to the IGFBP target of choice by 5' endogenous nuclease activity of Amplitaq Gold DNA polymerase results in an increase in the detection of fluorescence of the reporter dye upon release from the quencher which is quantified at each PCR cycle. A total reaction volume of 25 µl consisted of 200 nM forward primer (IGFBP-2,-3 or -4), 200 nM reverse primer (IGFBP-2, -3 or -4), 100 nM fluorescent probe for IGFBP-3 and -4, 200 nM fluorescent probe for IGFBP-2, 12.5 µl of Taqman Master mix without UNG, 0.625 µl Multiscribe

and RNase inhibitor mix (P/N 4309169, Applied Biosystems, Foster City, CA) and 100 ng of total RNA brought to volume with RNase free water. One-step RT-PCR amplification was performed in the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: 30 min at 48° C for reverse transcription, 95° C for 10 min for Amplitaq Gold activations, finishing with 40 cycles of 95° C for 15 sec for denaturing and 60° C for 1 min for annealing and extension. Ribosomal 18S RNA control kit (P/N 4308329, PE Biosystems, Foster City, CA) was used as a housekeeping gene to normalize samples for any variation in RNA loading. To verify that the 18S rRNA had the ability to detect fold changes in amounts of RNA loaded in a parallel fashion with the target probe (IGFBP-2, -3 or -4), decreasing amounts of total RNA were analyzed for 18S rRNA (500, 100, 50, 10, 5, or 1 pg) or target (IGFBP-2, -3 or -4) mRNA (500, 100, 50, 10, 5 or 1 ng) with results illustrated in Fig. 1.

Quantification of gene expression was made by setting an arbitrary threshold on the TET curves in the geometric portion of the RT-PCR amplification plot (Figure 2) after examining the log view. Relative quantification of IGFBP-2, -3 and -4 mRNA expression was done using the comparative threshold cycle (Ct) method (ABI SDS User Bullentin #2). Briefly, the ΔCt was determined by subtracting the 18S Ct from the target unknown (IGFBP-2, -3 or -4) Ct value. For each IGFBP mRNA, the ΔΔCt was determined by subtracting the highest ΔCt from all other ΔCt values within each experiment. Fold changes in mRNA expression of IGFBP-2, -3 or -4 were then calculated as $2^{-\Delta\Delta Ct}$. For each mRNA species, data are expressed as fold of the lowest treatment-group mean fold gene expression within an experiment.

RT-PCR Primer and Probe Design. Primers and probes for quantitative RT-PCR were made using Primer Express™ software (Foster City, CA) with the following manufacture's restrictions: The temperature melting (Tm) for primers was set at 50 to 60^0 C with the probe's Tm to be at least 10° C higher. The minimum GC bp content for the primers and probes should be 20-80% avoiding runs of an identical nucleotide. The minimum length of the strands should be 9 nucleotides though not to exceed 40.

The available bovine sequences for IGFBP-2, -3 and -4 in GENBANK (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) were analyzed by the Primer Express™ program to determine optimum primer and probe locations. IGFBP-2 (954 bp; Accession AF074854) forward and reverse primers were constructed from bp 466 to 486 with a sequence of GACGGGAACGTGAACTTGATG and from bp 518 to 536 with a sequence of TCCTTCATGCCGGACTTGA, respectively (Table 1). The probe for IGFBP-2 was found between bp 489 to 509 and has a sequence of AGGTGGAGGTGGTGCCGGTCG (Table 1). Forward and reverse primers for IGFBP-3 (1568 bp; Accession M76478) were designed from bp 1137 to 1163 with a sequence of AAAGAGATGTTTGAAATGCCTAGTTTT, and from bp 1200 to 1224 with a sequence of TCAAACTCGGTTTCACTGACTACTG, respectively (Table 1). The probe for IGFBP-3 was found between bp 1165 and 1191 has a sequence of TTCCACATGGTGAACCTGGCATCTTC (Table 1). An IGFBP-4 (2028 bp; Accession S52770) forward primer was constructed from bp 1733 to 1757 with a nucleotide sequence of GAGGAAAGAATGTATGTGCCTGATG while the reverse primer was found between bp 1808 to 1827 with a sequence of GACCACAAACGGAGGAGGAA (Table 1). The IGFBP-4 probe was located from bp 1772 to 1799 with the sequence of

CATGCTGGGAGGTGAGGGACTTATCTGG (Table 1). All primers and probes met aforementioned specifications regarding Tm and GC content set forth by the manufacturer. A blast search (http://www.ncbi.nlm.nih.gov/BLAST/) was also conducted to assure that primers and probes had not been designed from any homologous regions that would code for other proteins.

STATISTICAL ANALYSES

Data are presented as the least-squares means ± SEM. Each experiment was replicated 3 to 4 times with different pools of granulosa and theca cells, with duplicate samples taken from each pool. Each pool of theca cells and large-follicle granulosa cells was obtained from 5 to 7 follicles. Each pool of small-follicle granulosa cells was obtained from approximately 5 to 15 ovaries. For each experiment, treatments were applied in quadruplicate culture wells with each sample being obtained from two wells as described earlier. Using the general linear model of SAS (1999), treatment effects were determined. Specifically, main effects (treatments and experimental replicates) and interactions on dependent variables (IGFBP mRNA levels) were analyzed. Outliers were detected and determined by an outlier determination test as described by Ott (1977). IGFBP mRNA levels are expressed as relative mRNA abundance and specific differences in relative mRNA abundance were determined using Fischer's protected least significant difference test (Ott, 1977) if significant treatment effects were detected.

RESULTS

Experiment 1: Effects of estradiol, FSH and (or) LH on small-follicle granulosa IGFBP mRNA levels.

Small-follicle granulosa cells had detectable amounts of mRNA for IGFBP-2, -3 and -4. Estradiol, FSH and LH treatments had no effect ($P > 0.30$) on IGFBP-2 or -4 mRNA levels in granulosa cells from small-follicles. However, both FSH and LH increased ($P < 0.05$) IGFBP-3 mRNA levels when compared to control values (Fig. 3B). In contrast, estradiol had no effect ($P > 0.10$) on basal and FSH-induced IGFBP-3 mRNA levels but reduced the LH-induced increase in IGFBP-3 mRNA levels such that control values and LH plus estradiol did not differ (Fig. 3B). The target Ct and ΔCt values for Experiment 1 are summarized in Table 1.

Experiment 2: Effects of estradiol, FSH, and (or) LH on small-follicle granulosa IGFBP mRNA levels in the presence of insulin

As in Experiment 1, all three IGFBPs were detected by fluorescent real-time quantitative RT-PCR in these treated cells, but no effect ($P > 0.10$) of estradiol, FSH and LH on IGFBP-2 or -4 mRNA levels in small-follicle granulosa cells was detected. However, FSH treatment at both 3 and 30 ng/ml stimulated IGFBP-3 mRNA levels by 2- to 3-fold, respectively when compared to insulin-treated controls (Fig. 4B). However, neither LH nor estradiol (3 or 300 ng/ml) affected ($P > 0.10$) IGFBP-3 mRNA levels in the presence of insulin. The target Ct and ΔCt values for Experiment 2 are summarized in Table 1.

Experiment 3: Effects of IGF-1 and IGF-2 on small-follicle granulosa cell IGFBP mRNA levels

The effects of 100 ng/ml of IGF-1 and IGF-2 are shown in Figure 5. In Experiment 3, 24-h treatment of 100 ng/ml of IGF-1 had no effect ($P > 0.10$) on mRNA levels for IGFBP-2, -3 or -4. Similarly, treatment with 100 ng/ml of IGF-2 for 24 h had

no effect ($P > 0.10$) on IGFBP-2, -3 or -4 mRNA levels in granulosa cells from small-follicles. The target Ct and ΔCt values for Experiment 3 are summarized in Table 3.

Experiment 4: Effects of insulin, FSH, LH and estradiol on large-follicle granulosa cell IGFBP mRNA levels

IGFBP-2, -3 and -4 mRNA were all produced at detectable levels in large-follicle granulosa cells. Insulin, estradiol, FSH and LH treatments had no effect ($P > 0.25$) on IGFBP-3 or IGFBP-4 mRNA levels in large-follicle granulosa cells. In contrast, treatment with insulin alone increased ($P < 0.05$) relative IGFBP-2 mRNA levels when compared to controls (Fig. 6A). However, treatment with FSH and LH plus insulin had no further effect ($P > 0.10$) on insulin-stimulated IGFBP-2 mRNA levels. Furthermore, treatment with 3 or 300 ng/ml of estradiol along with FSH plus insulin did not affect ($P > 0.10$) IGFBP-2 mRNA levels when compared to treatment with FSH plus insulin alone (Fig. 6A). The target Ct and ΔCt values for Experiment 4 are summarized in Table 2.

Experiment 5: Effects of IGF-1, IGF-2, FSH, and leptin on relative abundance of IGFBP mRNA in large-follicle granulosa cells

Treatment with IGF-1, IGF-2, FSH or leptin did not affect ($P > 0.10$) IGFBP-3 or -4 mRNA levels in granulosa cells from large follicles. In contrast, 100 ng/ml of IGF-1 tended ($P < 0.06$) to increase IGFBP-2 mRNA levels by 2-fold above control cultures (Fig. 7A). However, treatment with 3 ng/ml of IGF-1, FSH, leptin or 100 ng/ml of IGF-2 had no effect ($P > 0.10$) on IGFBP-2 mRNA levels when compared to basal levels produced by control cultures. The target Ct and ΔCt values for Experiment 5 are summarized in Table 3.

Experiment 6: Effects of insulin, estradiol, and LH on IGFBP mRNA levels in theca cells from large follicles

Theca cells from large follicles had detectable mRNA for IGFBP-2, -3 and –4. Treatment with insulin, estradiol or LH did not affect ($P > 0.10$) mRNA levels for IGFBP-2 (Fig. 8A). In contrast, significant treatment effects were observed for both IGFBP-3 ($P < 0.05$) as well as IGFBP-4 ($P < 0.001$) (Fig. 8B and 8C). Treatment with estradiol decreased ($P < 0.05$) IGFBP-3 levels in theca cells (Fig. 8B). Treatment with estradiol plus insulin caused a similar decrease in IGFBP-3 mRNA levels as observed with estradiol alone (Fig. 8B). Treatments with LH, LH plus insulin, and insulin alone had no effect ($P > 0.10$) on IGFBP-3 mRNA levels (Fig. 8B). However, IGFBP-4 mRNA levels were decreased ($P < 0.05$) by treatments with insulin alone and estradiol alone compared to control cultures; these inhibitory effects were not altered by concomitant treatment with other hormones (Fig. 8C). Treatment with LH alone had no effect ($P > 0.10$) on IGFBP-4 mRNA levels in theca cells. The target Ct and ΔCt values for Experiment 6 are summarized in Table 2.

Experiment 7: Effects of IGF-1 and IGF-2 on IGFBP mRNA levels in theca cells from large follicles

In Experiment 7, no treatment effects ($P > 0.10$) were detected for IGFBP-3 or –4 when treated with 100 ng/ml of IGF-1 or IGF-2 (Fig. 9). However, treatment with 100 ng/ml of IGF-2 caused a 2.5-fold decrease ($P < 0.05$) in IGFBP-2 mRNA levels when compared to controls. IGF-1, on the other hand, had no effect ($P > 0.10$) on IGFBP-2 mRNA levels in theca cells (Fig. 9). The target Ct and ΔCt values for Experiment 7 are summarized in Table 3.

DISCUSSION

The results of the present study revealed that: 1) IGFBP-2, -3 and -4 mRNAs were all readily detectable by fluorescent real-time quantitative RT-PCR in granulosa cells from large and small follicles as well as theca cells from large follicles. 2) Insulin and estradiol decreased IGFBP-4 mRNA levels, while estradiol decreased IGFBP-3 mRNA levels and IGF-2 decreased IGFBP-2 mRNA levels in theca cells. 3) Insulin and IGF-1 increased large-follicle granulosa cell IGFBP-2 mRNA levels, whereas estradiol, LH, leptin and IGF-2 did not affect IGFBP-2, -3 or -4 mRNA levels in large-follicle granulosa cells. 4) FSH and LH increased IGFBP-3 mRNA levels in small-follicle granulosa cells whereas hormone treatments did not affect IGFBP-2 or -4 mRNA levels in granulosa cells obtained from small follicles.

In the present study we detected mRNA for IGFBP-2, -3 and -4 in both theca and granulosa cell cultures using fluorescent quantitative real-time RT-PCR. Previously, using RT-PCR, IGFBP-2 was predominately found in granulosa cells while IGFBP-4 was found in thecal cell cultures, with IGFBP-3 detected in large-follicle granulosa and theca cells in cattle (Armstrong et al., 1998; Voge et al., 2001). Using in situ hybridization in bovine follicles, IGFBP-2 mRNA was primarily found to be expressed in the granulosa cells of subordinate follicles, while IGFBP-3 was detected in only 2 of 28 follicles examined (Yuan et al., 1998). In healthy ovine ovarian follicles, IGFBP-2 mRNA was found to be localized in granulosa cells, while IGFBP-4 and IGFBP-5 mRNA were mainly expressed by thecal cells (Besnard et al., 1996). Similarly in porcine ovaries,

IGFBP-2 mRNA was localized to granulosa cells and IGFBP-4 to theca cells with low amounts of IGFBP-3 detected in both cells types (Liu et al., 2000; Wandji et al., 2000). Differences in studies in localization and abundance of detected IGFBP mRNA may in part be explained by sensitivity of the technique used, physiological status of the follicle, and species examined.

FSH effects on IGFBP mRNA

In the present study, 24-h treatment with FSH had no effect on IGFBP-2 or IGFBP-4 mRNA levels in granulosa cells of small and large follicles. Similarly, FSH had no effect on IGFBP-2/5 or IGFBP-4 production by cultured bovine (Chamberlain and Spicer, 2001), human (Cataldo et al., 1993) or ovine (Armstrong et al., 1996) granulosa cells. However, in cultured bovine granulosa cells collected from medium-sized follicles (4-8 mm) treated with 50 ng/ml of FSH for 4 d, IGFBP-2 mRNA levels were significantly decreased while 1 ng/ml was without effect (Armstrong et al., 1998). In the present study, FSH-induced increases in IGFBP-3 mRNA levels in small-follicle granulosa cells but had no effect on large-follicle granulosa cell IGFBP-3 mRNA. Similarly, Ingman et al. (2000) reported that bovine granulosa cells associated with the oocyte-cumulus complex in small follicles showed an increase in IGFBP-3 production in vitro after 24 h exposure to FSH. Perhaps FSH increases local production of IGFBP-3 in small-follicle granulosa cells to increase local stores of IGF-1 for enhanced granulosa cell proliferation as the follicle develops. In vivo treatment with exogenous FSH in cattle had no effect on IGFBP-3, -4 and -5 levels in follicular fluid of dominant and large estrogen-active follicles (Echternkamp et al., 1994; Rivera and Fortune, 2001), but either increased IGFBP-2, levels in large estrogen-active follicles after 5 days of treatment (Echternkamp

et al., 1994) or 2 days of treatment (Rivera and Fortune, 2001), respectively. In further support of the results of the present study, Grimes et al. (1994) reported that stimulation of porcine granulosa cells with FSH had no effect on IGFBP-4 mRNA expression. In rat granulosa cells, a biphasic effect of FSH on IGFBP-4 has been shown, with low doses (3 ng/ml) increasing and high doses (30 and 100 ng/ml) inhibiting production (Erickson et al., 1994). In FSH-treated rat granulosa cell cultures, two lower molecular weight bands (21.5 and 17.5 kDa) were detected with an IGFBP-4 antibody indicating the possibility of an FSH-induced IGFBP-4 protease (Liu et al., 1993). Recently, it has been suggested that decreases in IGFBP-4 could be due in part to increased levels of pregnancy associated plasma protein-A (PAPP-A), an IGFBP-4 specific protease produced by granulosa cells (Conover et al., 2001; Mazerbourg et al., 2001). Thus, this data taken together would suggest that species differences, variable dose and duration of FSH treatment, variation in the differentiated state of granulosa cells used, and (or) presence of IGFBP proteases may explain the differences among studies for the response to FSH.

Insulin effects on IGFBP mRNA

In the present study, insulin increased IGFBP-2 mRNA levels in large-follicle granulosa cells and decreased IGFBP-4 mRNA levels in thecal cells but had no effect on IGFBP-3 mRNA levels in either cell type. Consistent with the present study, treatment of cultured human (Greisen et al., 2002) and porcine (Grimes and Hammond, 1992) granulosa cells with insulin increased levels of IGFBP-2 in medium. However, in bovine large-follicle granulosa cells, insulin decreased a band on ligand blots that co-migrated with IGFBP-2/-5 but which specific binding protein was decreased could not be ascertained (Spicer and Chamberlain, 2002). Interestingly, in rat hepatocytes, insulin

inhibited IGFBP-2 production and levels of its mRNA (Schmid et al., 1992). Similar to the present study, insulin had no effect on IGFBP-3 production by human granulosa cells (Cataldo et al., 1993; Greisen et al., 2002) or bovine granulosa and theca cells (Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002). In contrast to the present study, 24-h insulin treatment did not affect IGFBP-4 protein production in bovine thecal cells (Spicer and Chamberlain, 2002). Many studies indicate that an up- or down-regulation in mRNA expression precede a change in detectable protein levels (LaPolt et al., 1990; Rosato et al., 2001; Wang et al., 2001), and may in part explain this latter discrepancy between studies. In addition, insulin's effect on IGFBP-4 may be dose dependent, as insulin at low doses ($\leq$ 10 ng/ml) had no effect while high doses ($\geq$ 100 ng/ml) increased IGFBP-4 in human endometrial cells (Irwin et al., 1995). Therefore, the effects of insulin on IGFBP production and mRNA levels may depend on cell type, species, dose of insulin used, and duration of treatment.

IGF effects on IGFBP mRNA

In the present study, levels of IGFBP-3 and -4 mRNA in granulosa and theca cells were not affected by IGF-1 or -2. However, treatment with IGF-1 tended to increase IGFBP-2 mRNA levels in large-follicle granulosa cells, while having no effect on IGFBP-2 mRNA levels either theca or small-follicle granulosa cells. In contrast, IGF-2 decreased IGFBP-2 mRNA levels in bovine theca cells in the present study. Similarly, Cataldo et al. (1993) reported a 49% decrease in IGFBP-2 levels in human luteinizing granulosa cells after treatment with IGF-2. Moderately differentiated porcine granulosa cells had increased levels of IGFBP-3 and IGFBP-2 as well as increased IGFBP-3 mRNA levels after 48 h exposure to IGF-1 (Grimes and Hammond, 1992; Grimes et al., 1994).

IGFBP-3 mRNA levels were increased to a maximum after 6 h of treatment with IGF-1 and remained elevated after 24 h in bovine fibroblasts (Bale and Conover, 1992). In further support of the present study, IGFBP-3 production by bovine small-follicle granulosa cells did not change after 24 h treatment with IGF-1 (Ingman et al., 2000). Perhaps the overall role of IGFs on IGFBP production in the bovine ovary may be negligible. Direct infusion of IGF-1 into the ovarian interstitium in cattle had no effect on levels of IGFBPs in follicular fluid of the two largest follicles when compared to animals receiving saline (Spicer et al., 2000). In human endometrial cells, IGF-1 showed decreased levels of IGFBP-1 and IGFBP-4 with no effect on IGFBP-2 levels, while IGF-2 decreased levels of IGFBP-1, -2 and -3 (Irwin et al., 1995). Through a different mechanism than FSH, IGF-1 may also be decreasing levels of IGFBP-4 found in follicular fluid through induction of IGFBP-4 protease activity. For example, when IGF-1 was added to rat granulosa cell cultures it activated the proteolysis of radiolabelled IGFBP-4 (Iwashita et al., 1998). Furthermore, addition of LR3-IGF-1, which does not bind IGFBP-4, did not result in cleavage of IGFBP-4 indicating that IGF-1 must bind IGFBP-4 to expose its cleavage site to the protease (Iwashita et al., 1998). This is further substantiated, in that addition of IGFBP-2 and -3, which compete with IGFBP-4 for IGF-1, along with IGF-1 to media containing IGFBP-4 and bovine follicular fluid decreased IGFBP-4 proteolytic activity (Mazerbourg et al., 2000). However, this affect was removed upon addition of excess IGF-1 to culture (Mazerbourg et al., 2000) Huynh (2000) postulates the role of IGF-1 in rat myometrial IGFBP-4 production may be to stabilize the mRNA, providing a constant source of IGFBP-4 protein, with decreasing protein levels found in media attributed to an increase in protease activity. Thus, whether

or not IGFs have an effect on IGFBP mRNA levels or production may depend on many factors including species, cell type, and which specific IGF receptor is present in a particular cell type. Also, IGF-1 may be inducing specific protease degradation of IGFBP-4 by binding to IGFBP-4, thus allowing for more free IGF-1 to be available to promote further ovarian function.

Estradiol effects on IGFBP mRNA

The present study found that estradiol had no effect on IGFBP-2 mRNA levels in any cell type, while reducing the stimulatory effect of LH and FSH on IGFBP-3 mRNA in small-follicle granulosa cells and decreasing mRNA levels of IGFBP-3 and -4 in thecal cells of large follicles. Consistent with our study, Mondschein et al. (1990) reported that 3-day estradiol treatment has no effect on IGFBP-2 or -3 production by porcine granulosa cells. Also, treatment with diethylstilbestrol to hypophysectomized rats decreased IGFBP-3 transcript levels in the theca-interstitial layer of the ovary (Ricciarelli et al., 1992). Similar to the inhibitory effect of estradiol on thecal IGFBP-3 mRNA, MCF-7 cells cultured for 96 h with 10 nM estradiol had reduced IGFBP-3 levels (25% decrease) when compared to controls (Martin et al., 1995). Because IGFBP-3 is the major IGFBP present in follicular fluid of cattle and IGFBP-3 levels remain relatively constant during follicular growth (de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996), it is unlikely that changes in local production or expression levels will contribute extensively to the overall amounts of IGFBP-3 in vivo. Instead, a decrease in locally produced IGFBP-3 may reduce the biological half-life of IGF-1 in or around the cells that are producing IGFBP-3. Similar to IGFBP-3 mRNA levels, estradiol decreased IGFBP-4 mRNA levels in cultured theca cells of the present study. In-situ hybridization studies in

porcine follicles have shown that IGFBP-4 mRNA localization in theca cells is at a minimum when estradiol levels in follicular fluid are at a maximum (Liu et al., 2000). Also, bovine granulosa cells from large and small follicles treated with estradiol for 24 h, produced less IGFBP-4 than control cultures but produced similar amounts of IGFBP-3 (Spicer and Chamberlain, 2002). In cultured osteoblastic cells, IGFBP-4 production was decreased by estrogen (Kudo et al., 1995; Kassem et al., 1996). In agreement with the present study, estradiol had no effect on bovine thecal cell IGFBP-2 production (Spicer and Chamberlain, 2002). Similarly, in MCF-7 cells, neither IGFBP-2 mRNA nor protein levels were affected by treatment with estradiol (Kim et al., 1991). However, rats treated with estrogens displayed increased levels of IGFBP-2 mRNA abundance in theca cells when compared to control animals (Riccarelli et al., 1991). Overall, the effects of estradiol on the ovary may be to stimulate growth through inhibition of IGFBP-4 mRNA expression and thus decreased IGFBP-4 production allowing for more bioaviable IGFs to the dominant follicle. This is further substantiated by the fact that IGFBP-2, -4 and -5 protein levels are negatively correlated with estrogen levels in bovine antral follicles in vivo (Stewart et al., 1996; Austin et al., 2001; Spicer et al., 2001).

LH effects on IGFBP mRNA

LH had no effect on mRNA levels for IGFBP-2 or IGFBP-4 in any cell type that was examined in the present study. However, IGFBP-3 mRNA levels were increased by LH in the absence of insulin in small-follicle granulosa cells. IGFBP-3 mRNA levels were not influenced by LH in theca or large-follicle granulosa cells. Similar to our present study involving IGFBP-2 mRNA levels, LH had no effect on IGFBP-2 production in rat (Erickson et al., 1995), human (Voutilainen et al., 1996), ovine

(Armstrong et al., 1996), or bovine (Chamberlain and Spicer, 2001) thecal cells in vitro. In human granulosa cells cultured in a polycystic ovary environment, 2-d treatment of LH had no effect on IGFBP-2 production (Greisen et al., 2002). However, 3-d treatment of 10 ng/ml hCG stimulated IGFBP-2 accumulation in luteinized human granulosa cells from normal ovaries (Giudice et al., 1991), while 3-5 d treatment of 100 ng/ml of hCG decreased IGFBP-2 production in another study (Cataldo et al., 1993). In agreement with the present study, IGFBP-3 protein levels increased in bovine theca cells after 24 h treatment with 100 ng/ml of LH, however no effect of LH was seen on granulosa cell IGFBP-3 levels in the same study (Spicer and Chamberlain, 2002). In human thecal cells, IGFBP-3 production was blocked by 2-d treatment with 100 ng/ml of hCG (San Roman and Magoffin, 1992). In ovine thecal cells, LH caused a time- and dose-dependent increase in IGFBP-4 production with maximal production seen after 4- and 6-days in culture with 10 ng/ml of LH (Armstrong et al., 1996). In addition, IGFBP-4 mRNA levels in bovine thecal cells were increased by 100 ng/ml of LH after 4 day treatment (Armstrong et al., 1998). Similarly, in vivo a subcutaneous injection of hCG to PMSG primed immature rats resulted in a 3.5-fold increase in total ovarian IGFBP-4 mRNA levels 24 h post-injection (Putowski et al., 1997). This is in contrast to the present study where no changes were observed in either granulosa or theca cell IGFBP-4 mRNA levels after 24 h exposure to 30 ng/ml of LH. Discrepancies between this study and others on the effects of LH on ovarian IGFBP-2, -3 and -4 mRNA levels and production may in part be explained by species differences, as well as differences in culture conditions (i.e. length of time in culture) and differences in concentrations of LH used, the latter two of which may influence whether cultured cells luteinize during

treatment. Collectively, conditions that would cause cells to luteinize in vitro (i.e., extended periods of high LH/hCG doses) appear to decrease granulosa cell IGFBP-2 and increase theca cell IGFBP-4 production.

Leptin had no effect on IGFBP-2, -3 or -4 mRNA levels in bovine large-follicle granulosa cells. This is the first evidence as to whether or not leptin is influencing ovarian IGFBP gene expression. The lack of effect of leptin on IGFBP mRNA is consistent with previous findings that showed no effect of leptin on IGF-1-induced steroidogenesis of large-follicle granulosa cells (Spicer et al., 2000). Instead, leptin inhibits insulin-induced increases in steroid production by bovine granulosa cells (Francisco and Spicer, 1997). Overall, leptin is not affecting ovarian function in cattle through changes in IGFBP-2, -3 or -4 mRNA expression levels

The results from the current and previous studies indicate that IGFBP gene expression and thus production in the bovine ovary is regulated through a network of hormonal events. In the dominant follicle with levels of lower molecular weight IGFBP decreasing with follicular growth and increasing with atresia, IGFBP gene expression and (or) production are likely regulated through the dynamic hormonal changes that are occurring during a normal estrous cycle. A decrease in IGFBP gene expression and thus decrease in IGFBP production would lead to an increase in total amount of bioavailable IGFs allowing for increased growth and function of the dominant follicle. An increase in IGFBP gene expression and thus increase in IGFBP production would lead to a decrease in bioavailable IGFs preventing or slowing follicular growth and (or) inducing atresia.

Figure 1. Results of the test for parallelism between the housekeeping gene 18S rRNA (+) and IGFBP-2 (Δ, Panel A), IGFBP-3 (0, Panel B) or IGFBP-4 (◊, Panel C). Different fold amounts of total RNA (1 to 500 ng for IGFBPs and 1 to 500 pg for 18S rRNA) were added to PCR reaction as described in Materials and Methods to determine if fold changes in target could be detected in a parallel fashion.

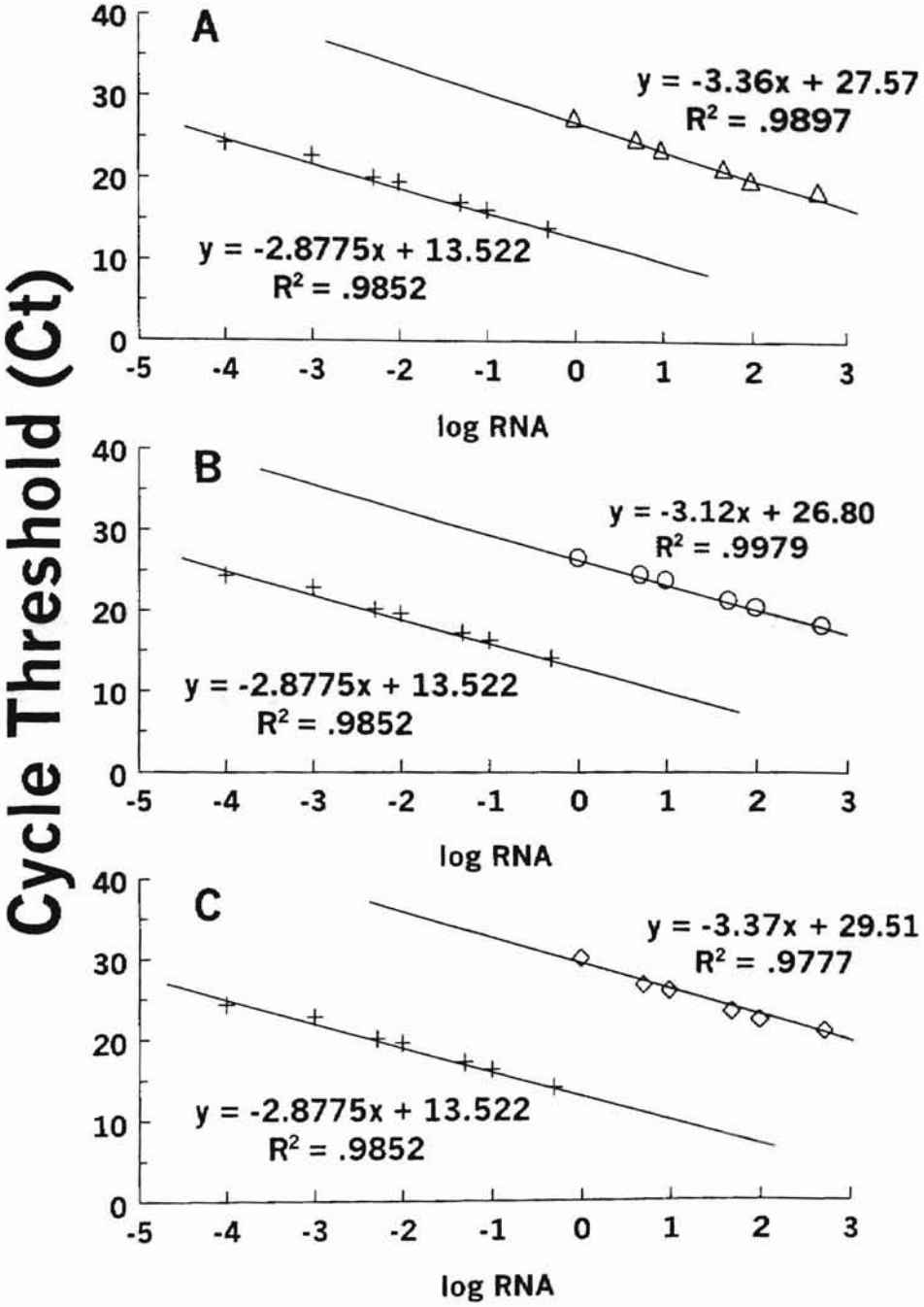
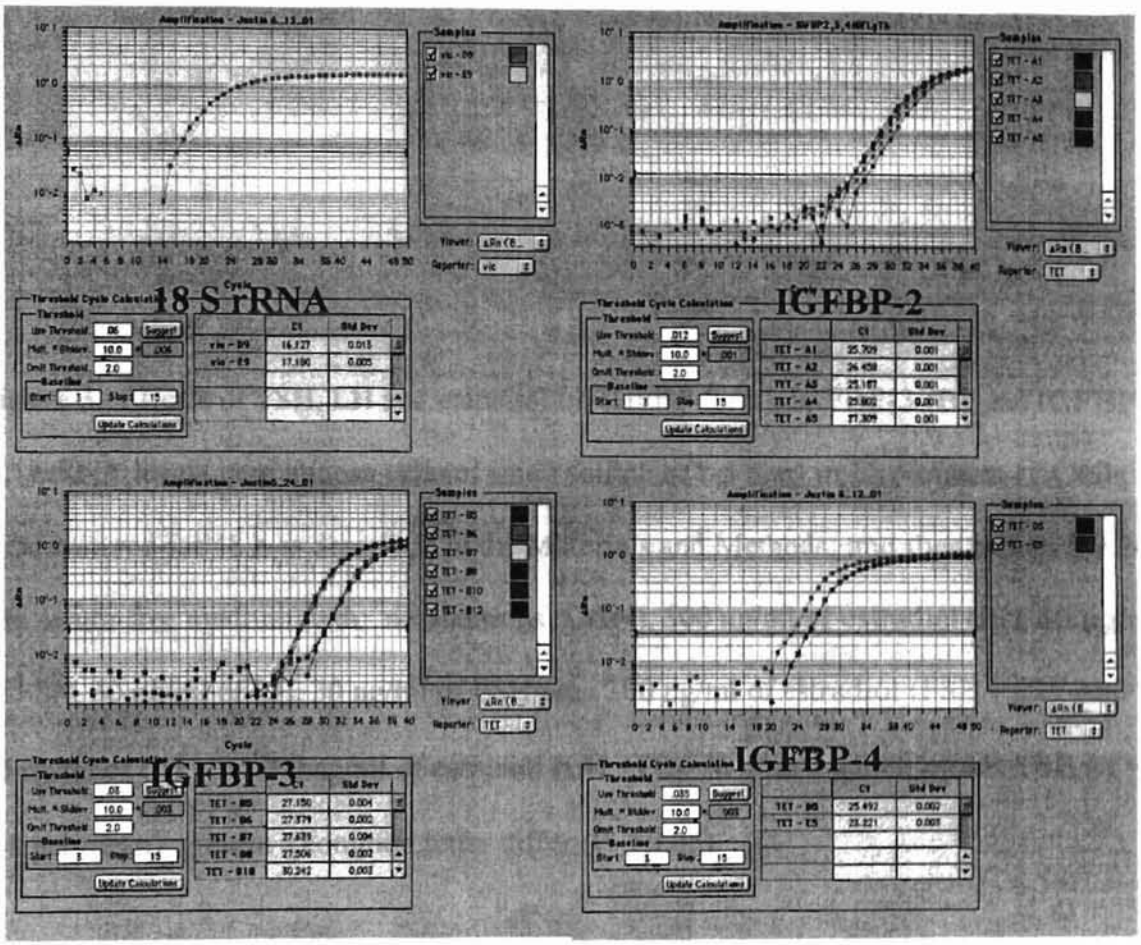

Cycle Threshold (Ct)
A
y = -3.36x + 27.57
R² = .9897
y = -2.8775x + 13.522
R² = .9852
log RNA
B
y = -3.12x + 26.80
R² = .9979
y = -2.8775x + 13.522
R² = .9852
log RNA
C
y = -3.37x + 29.51
R² = .9777
y = -2.8775x + 13.522
R² = .9852
log RNA
40
30
20
10
0
-5
-4
-3
-2
-1
0
1
2
3

Figure 2. Amplification plots for amounts of IGFBP-2, -3 and -4 mRNA and 18S rRNA from cultured bovine granulosa and theca cells using quantitative real-time rt-PCR.


18 S rRNA
IGFBP-2
IGFBP-3
IGFBP-4

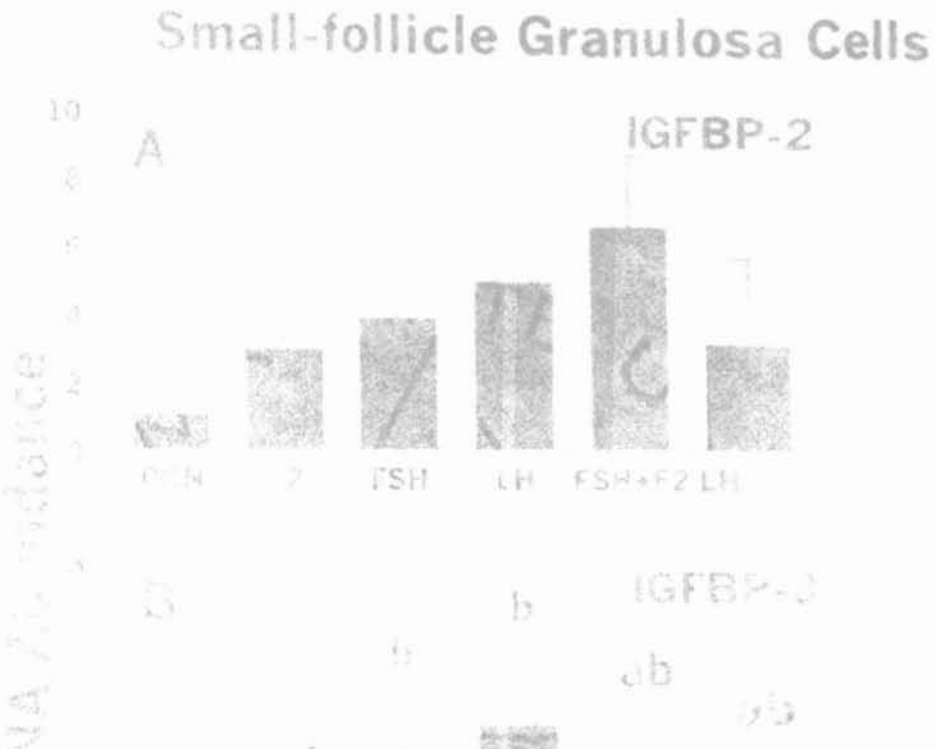


Figure 3. Effects of FSH, LH and estradiol on IGFBP-2 (A), IGFBP-3 (B), and IGFBP-4 (C) mRNA levels in granulosa cells of small follicles (1-5 mm) in Experiment 1. Cells were cultured for 48 h as described in the Materials and Methods, and then treated for 24 h in serum-free medium with: no additions (CON), 300 ng/ml of estradiol (E2), 30 ng/ml of FSH, 30 ng/ml of LH, 30 ng/ml of FSH plus 300 ng/ml of estradiol (FSH + E2), and 30 ng/ml of LH plus 300 ng/ml of estradiol (LH +E2). Within a panel, means ± SE (n = 4-8 per mean) without a common letter differ ($P < 0.05$).

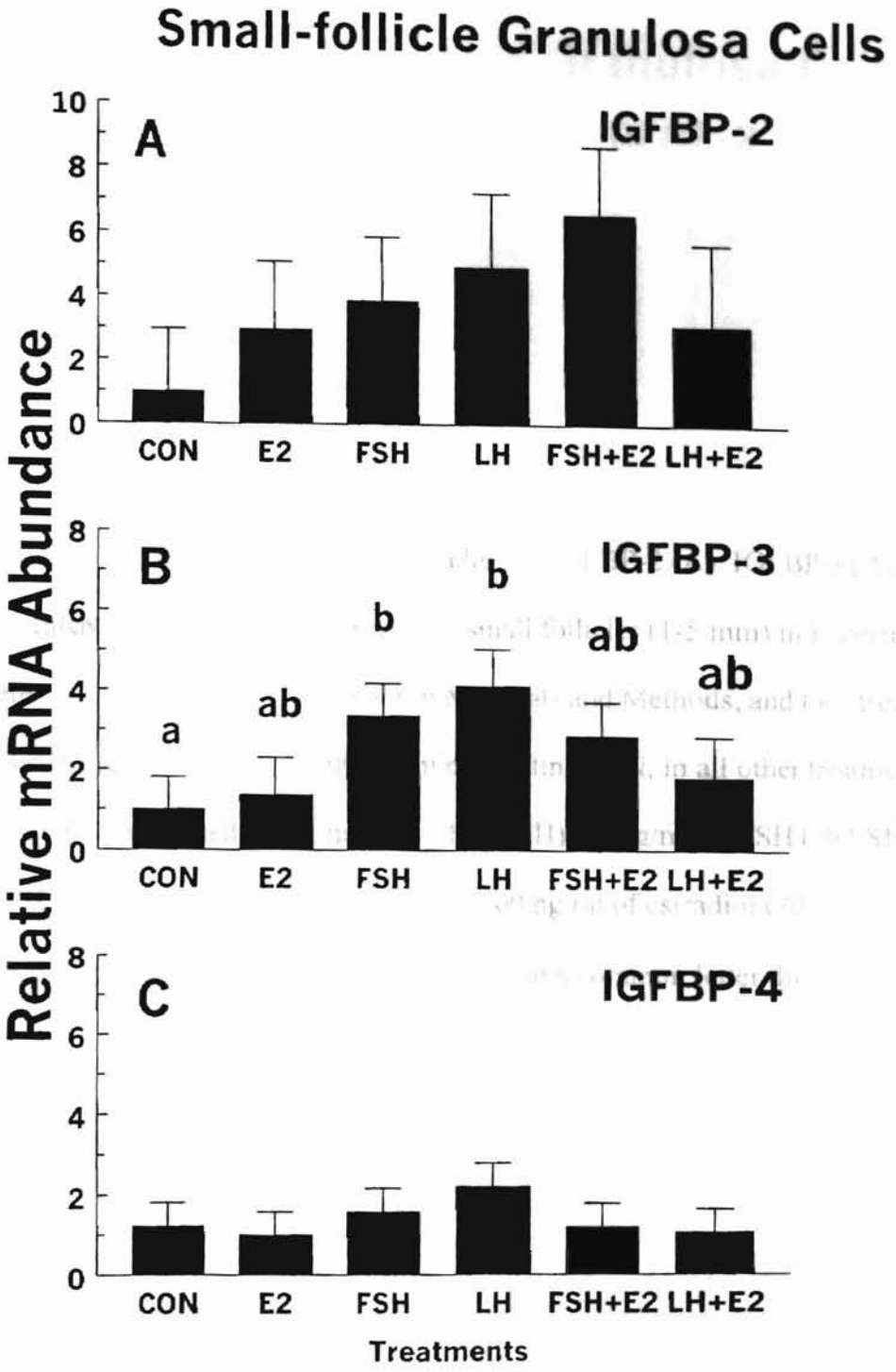
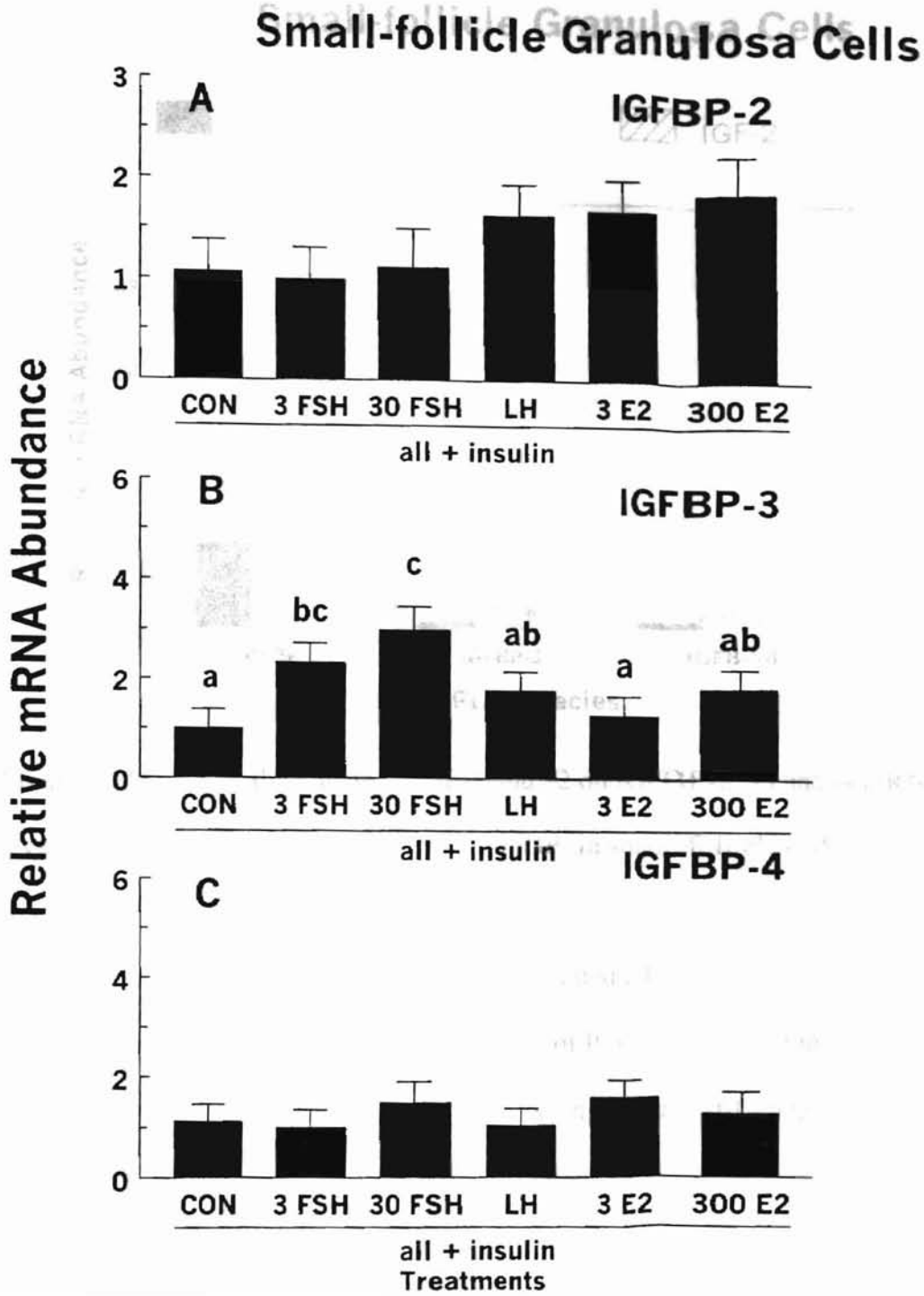

Small-follicle Granulosa Cells
A
IGFBP-2
B
IGFBP-3
C
IGFBP-4
Relative mRNA Abundance
0
2
4
6
8
10
a
ab
b
b
ab
ab
CON
E2
FSH
LH
FSH+E2
LH+E2
Treatments

Figure 4. Effects of insulin, FSH, LH and estradiol on IGFBP-2 (A), IGFBP-3 (B), and IGFBP-4 (C) mRNA levels in granulosa cells of small follicles (1-5 mm) in Experiment 2. Cells were cultured for 48 h as described in Materials and Methods, and then treated for 24 h in serum-free medium with 100 ng/ml of insulin (CON, in all other treatment groups unless otherwise stated), 3 ng/ml of FSH (3 FSH), 30 ng/ml of FSH (30 FSH), 30 ng/ml of LH (LH), 3 ng/ml of estradiol (3 E2), or 300 ng/ml of estradiol (300 E2). Within a panel, means ± SE (n = 4-8 per mean) without a common letter differ ($P < 0.05$).


Small-follicle Granulosa Cells
A
IGFBP-2
B
IGFBP-3
C
IGFBP-4
Relative mRNA Abundance
0
1
2
3
4
6
a
bc
c
ab
a
ab
CON
3 FSH
30 FSH
LH
3 E2
300 E2
all + insulin
Treatments

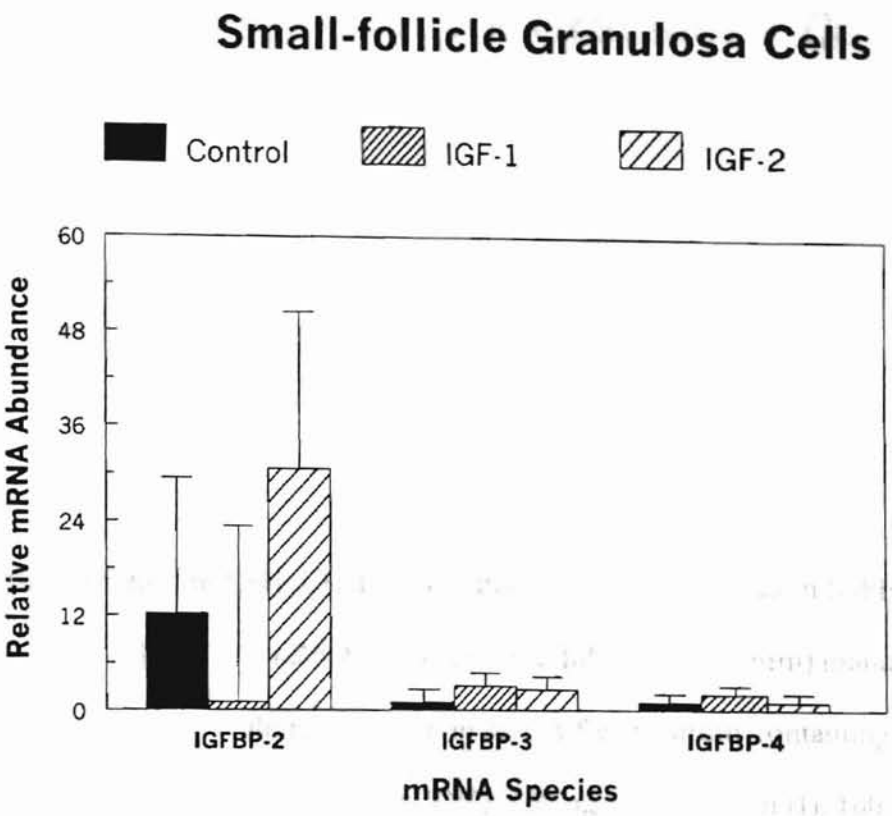


Figure 5. Results of the effects of IGF-1 and –2 on IGFBP-2, -3 and -4 mRNA levels (fold change in expression within IGFBP) in small-follicle (1-5 mm) granulosa cells of Experiment 3. Granulosa cells were cultured for 48 h as described in Materials and Methods and then treated 24 h in serum-free medium containing: no additions (Control), 100 ng/ml of IGF-1 or 100 ng/ml of IGF-2. Values are means ± SE (n = 4-8 per mean). No significant differences were detected ($P > 0.10$).

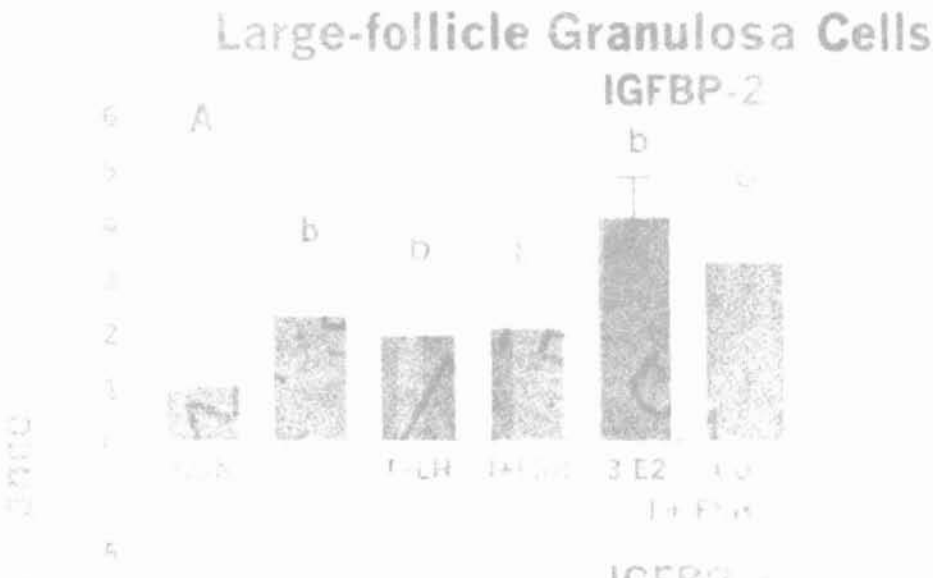
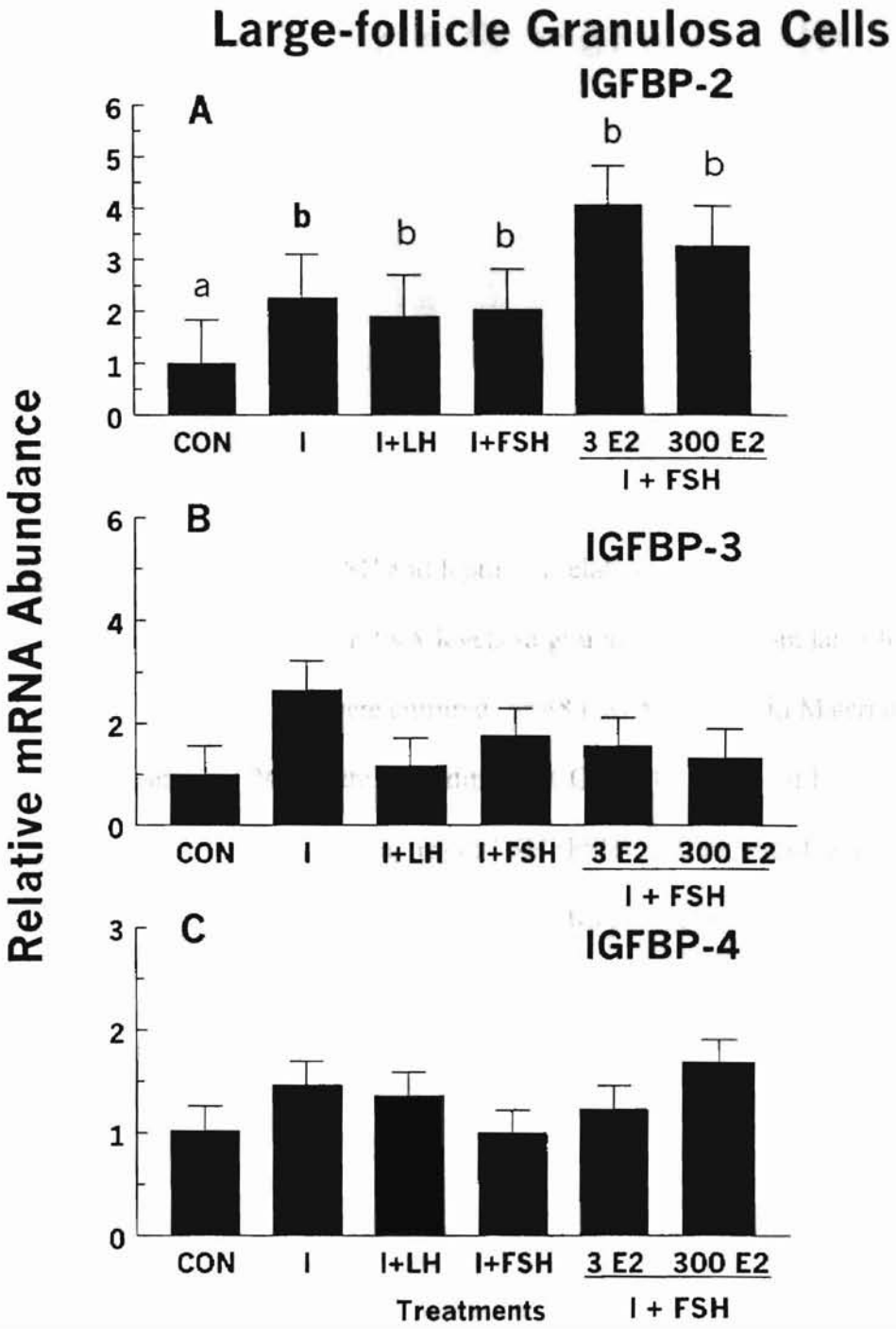


Figure 6. Effects of insulin, FSH, LH and estradiol on relative changes in IGFBP-2 (A), IGFBP-3 (B), and IGFBP-4 (C) mRNA levels in large-follicle (> 7.9 mm) granulosa cells of Experiment 4. Cells were cultured for 24 h in serum-free medium containing one of the following six treatments: no additions (CON), 100 ng/ml of insulin (I), 100 ng/ml of insulin plus 30 ng/ml of FSH (I + FSH), 100 ng/ml of insulin plus 30 ng/ml of LH (I + LH), 100 ng/ml of insulin plus 30 ng/ml of FSH plus 3 ng/ml of estradiol (3 E2), or 100 ng/ml of insulin plus 30 ng/ml of FSH plus 300 ng/ml of estradiol (300 E2). Within a panel, means (n = 4-8 per mean) without a common letter differ ($P < 0.05$).


Large-follicle Granulosa Cells
IGFBP-2
A
B
IGFBP-3
C
IGFBP-4
Relative mRNA Abundance
a
b
b
b
b
b
CON
I
I+LH
I+FSH
3 E2
300 E2
I + FSH
Treatments

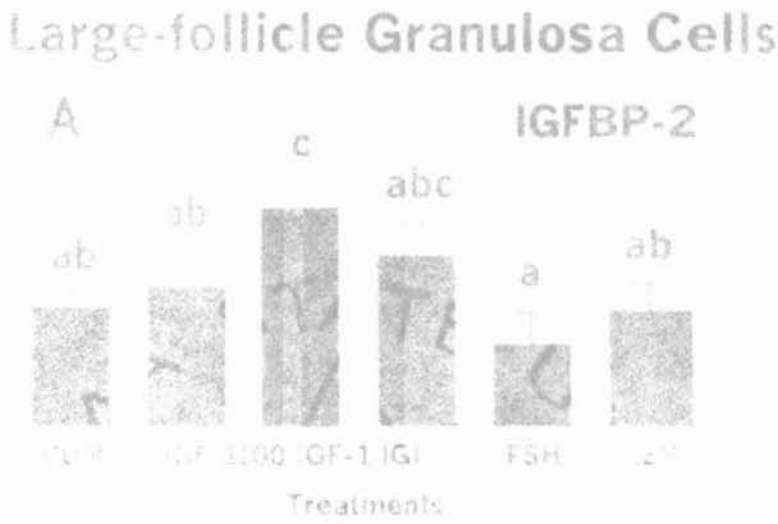
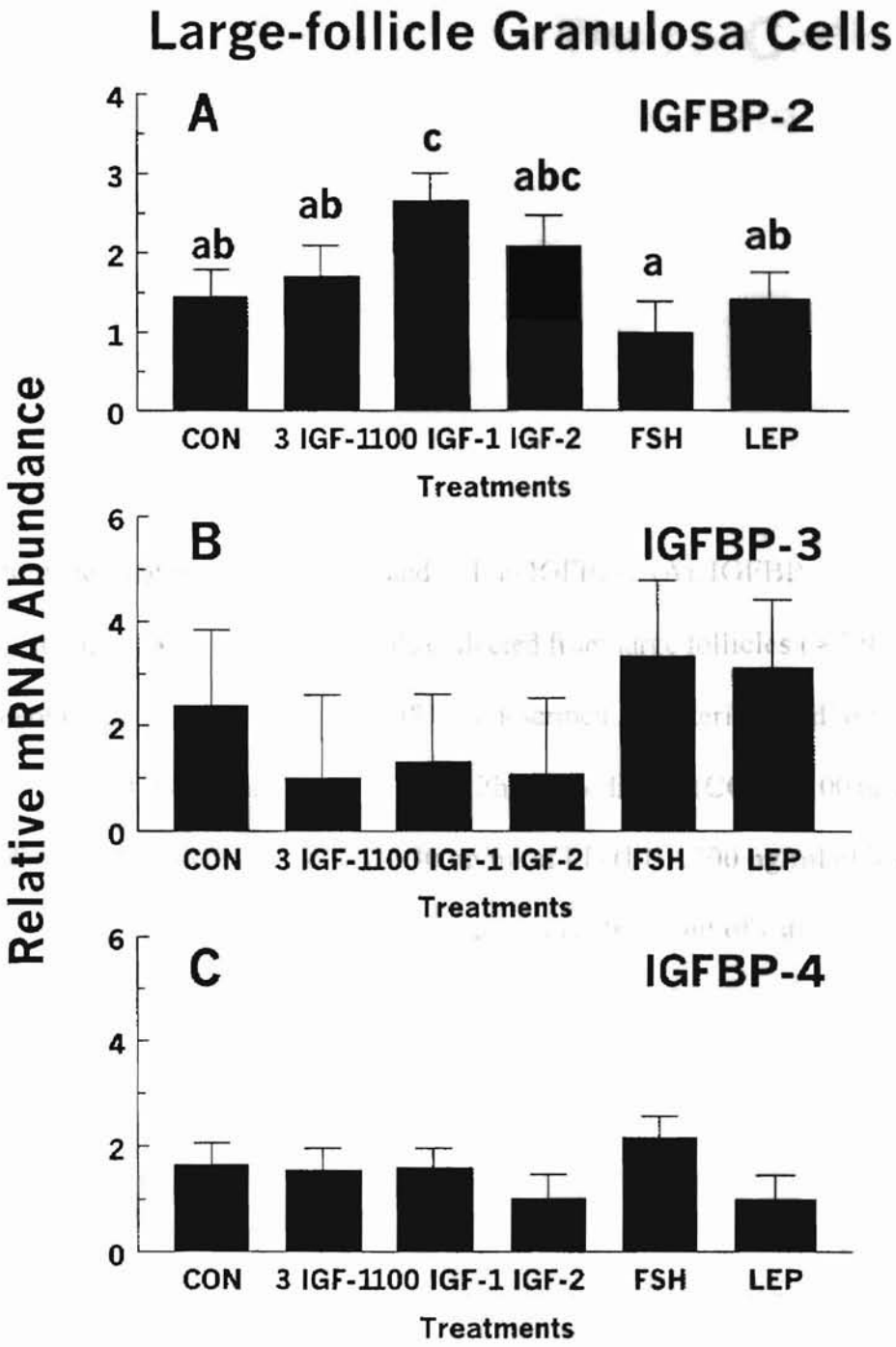


Figure 7. Effects of IGF-1, IGF-2, FSH and leptin on relative fold changes of IGFBP-2 (A), IGFBP-3 (B), and IGFBP-4 (C) mRNA levels in granulosa cells from large-follicles (> 7.9 mm) in Experiment 5. Cells were cultured for 48 h as described in Materials and Methods and then treated for 24 h with: no additions (CON), 100 ng/ml of IGF-1 (IGF-1), 100 ng/ml of IGF-2 (IGF-2), 3 ng/ml of FSH (FSH), 100 ng/ml of leptin (LEP), or 3 ng/ml of IGF-1. Within a panel, means ± SE (n = 4-8 per mean) without a common letter differ ($P < 0.06$).


Large-follicle Granulosa Cells
A
IGFBP-2
B
IGFBP-3
C
IGFBP-4
Relative mRNA Abundance
0
1
2
3
4
6
ab
ab
c
abc
a
ab
CON
3 IGF-1
100 IGF-1
IGF-2
FSH
LEP
Treatments

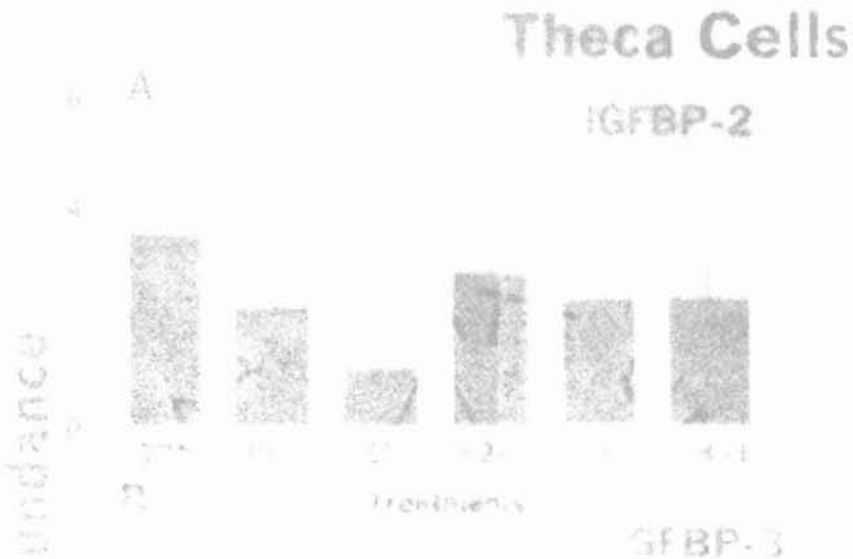
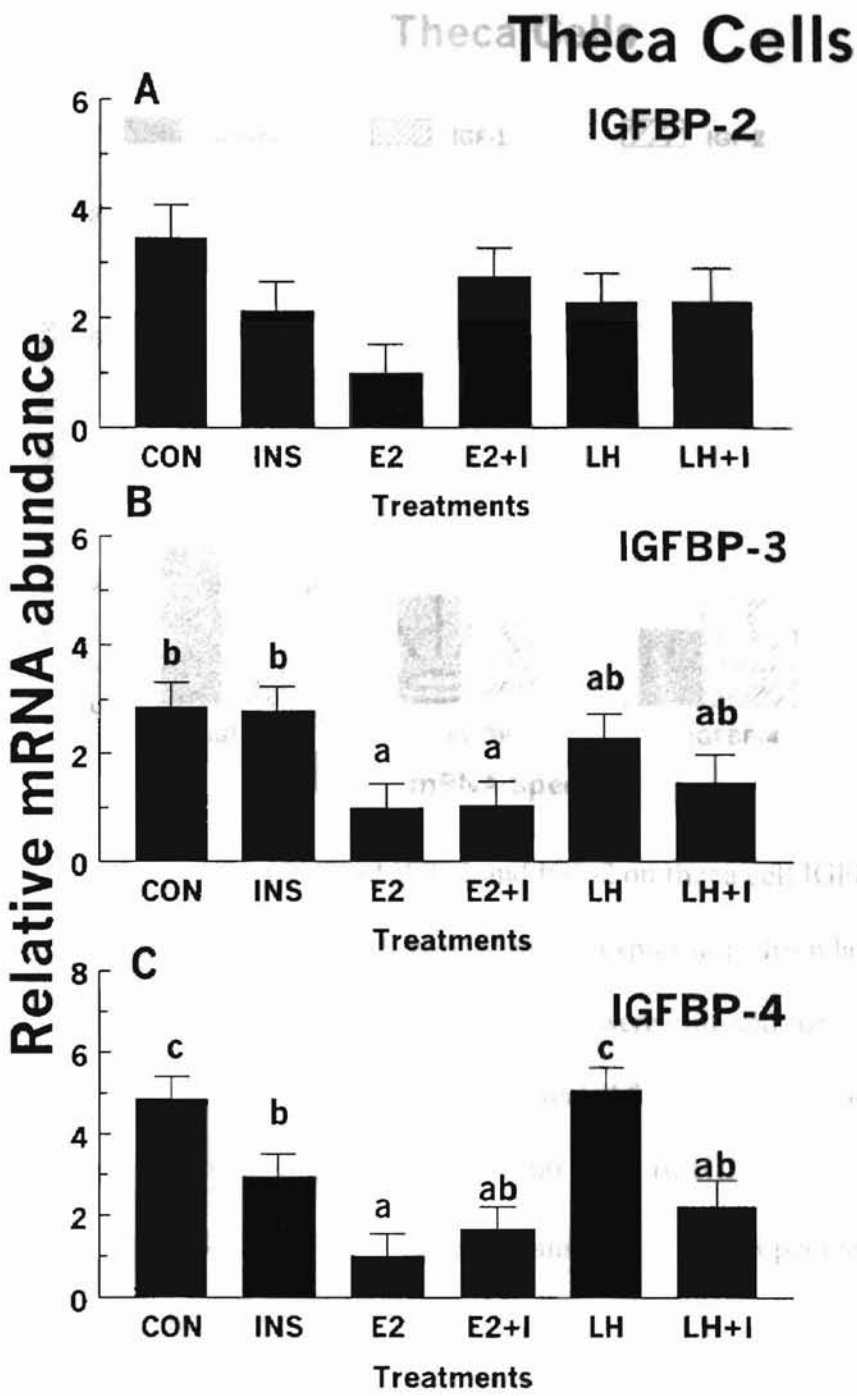


Figure 8. Effects of insulin, estradiol and LH on IGFBP-2 (A), IGFBP-3 (B), and IGFBP-4 (C) mRNA levels in theca cells collected from large follicles (> 7.9 mm) in Experiment 6. Cells were cultured for 48 h as described in Materials and Methods and then treated for 24 h in serum-free medium with: no additions (CON), 100 ng/ml of insulin (I), 500 ng/ml of estradiol (E2), 30 ng/ml of LH (LH), 100 ng/ml of insulin plus 30 ng/ml of LH (LH + I), or 100 ng/ml of insulin plus 500 ng/ml of estradiol (E2 + I). Within a panel means ± SE (n = 4-8 per mean) without a common letter differ ($P < 0.05$).


Theca Cells
A
IGFBP-2
B
IGFBP-3
C
IGFBP-4
Relative mRNA abundance
CON
INS
E2
E2+I
LH
LH+I
Treatments
a
b
c
ab

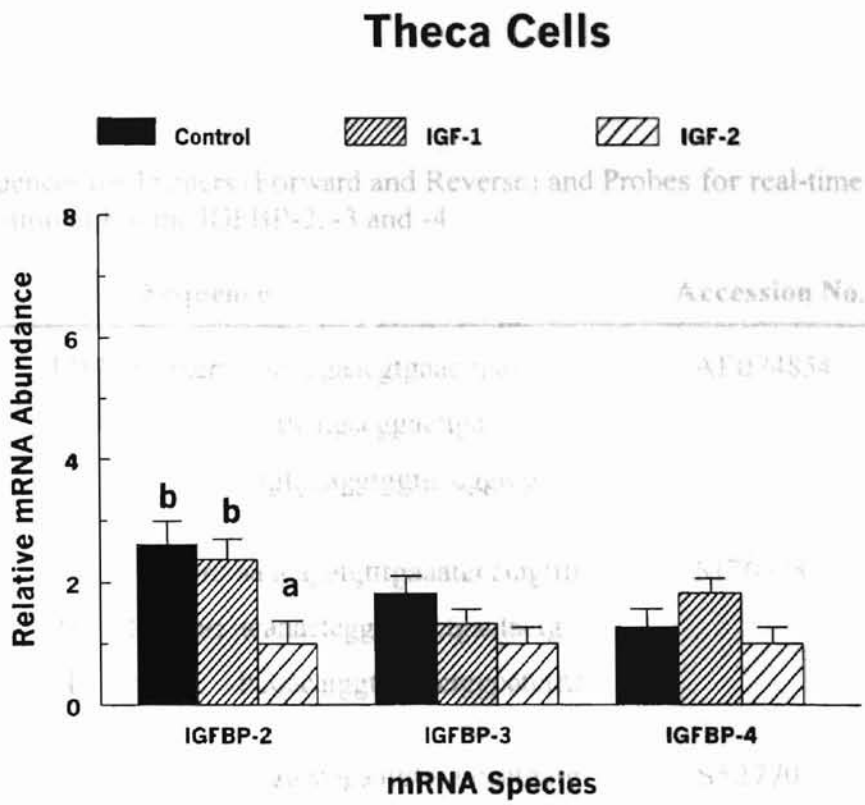


Figure 9. Results of the effects of IGF-1 and IGF-2 on theca cell IGFBP-2, -3 and –4 mRNA levels (relative fold change in gene expression) from large-follicles (> 7.9 mm) of Experiment 7. Theca cells were cultured for 48 h as described in Materials and Methods and then treated for 24 h in serum-free medium containing: no additions (Control), 100 ng/ml of IGF-1 or 100 ng/ml of IGF-2. Within an IGFBP mRNA species, means ± SE (n = 4-8 per mean) without a common letter differ ($P < 0.05$).

Table 1. Sequences for Primers (Forward and Reverse) and Probes for real-time RT-PCR amplification of bovine IGFBP-2, -3 and -4.

Gene	*Sequence	Accession No.
IGFBP-2	FWD Primer: gacgggaacgtgaacttgatg	AF074854
	REV Primer: tccttcatgccggacttga	
	TET Probe: aggtggaggtggtgccggtcg	
IGFBP-3	FWD Primer: aaagagatgtttgaaatgcctagtttt	M76478
	REV Primer: tcaaactcggtttcactgactactg	
	TET Probe: ttccacatggtgaacctggcatctttc	
IGFBP-4	FWD Primer: gaggaaagaatgtatgtgcctgatg	S52770
	REV Primer: gaccacaaacggaggaggaa	
	TET Probe: catgctgggaggtgagggacttatctgg	

Table 2. Quantitative PCR analysis of IGFBP-2, -3 and -4 mRNA expression in small-follicle granulosa cells treated with FSH, LH, and estradiol (E2) without (Exp. 1, Con) and with (Exp. 2, Con) 100 ng/ml of insulin (I).

	IGFBP-2			IGFBP-3			IGFBP-4		
Treatment	**BP-2 Ct[d]**	**18S Ct**	**ΔCt**	**BP-3 Ct[d]**	**18S Ct**	**ΔCt**	**BP-4 Ct[d]**	**18S C**	**ΔCt**
Exp. 1									
Con	29.7 ± 0.4	22.6 ± 0.8	7.1 ± 0.1	27.5 ± 0.5	20.6 ± 0.5	6.8 ± 0.5[a]	32.4 ± 1.1	21.2 ± 1.4	11.2 ± 0.9
E2	28.0 ± 0.5	21.5 ± 0.8	6.5 ± 0.7	26.3 ± 0.5	19.9 ± 0.6	6.4 ± 0.6[ab]	31.3 ± 1.1	19.2 ± 1.4	12.1 ± 0.9
FSH	28.3 ± 0.4	21.6 ± 0.8	6.6 ± 0.7	25.1 ± 0.5	20.3 ± 0.5	4.8 ± 0.5[b]	29.5 ± 0.9	19.2 ± 1.1	10.4 ± 0.7
LH	28.9 ± 0.5	22.6 ± 0.9	6.3 ± 0.8	26.8 ± 0.5	20.9 ± 0.6	5.9 ± 0.6[b]	30.3 ± 1.2	19.9 ± 1.5	10.3 ± 1.0
FSH + E2	28.8 ± 0.5	22.4 ± 0.8	6.4 ± 0.7	26.4 ± 0.5	20.3 ± 0.5	6.2 ± 0.5[ab]	30.1 ± 1.1	19.5 ± 1.4	10.7 ± 0.9
LH + E2	27.4 ± 0.6	20.7 ± 1.0	6.7 ± 0.9	26.1 ± 0.6	19.3 ± 0.6	6.8 ± 0.6[ab]	29.7 ± 1.4	18.2 ± 1.8	11.5 ± 1.2
Exp. 2									
Con + I	25.9 ± 0.2	16.3 ± 0.2	9.6 ± 0.3	22.0 ± 0.1	16.3 ± 0.3	5.7 ± 0.3[a]	28.0 ± 0.4	16.3 ± 0.2	11.7 ± 0.4
I + 3 FSH	26.2 ± 0.2	16.5 ± 0.2	9.7 ± 0.3	21.0 ± 0.1	16.5 ± 0.2	4.5 ± 0.3[bc]	28.1 ± 0.4	16.5 ± 0.2	11.6 ± 0.4
I + 30 FSH	25.9 ± 0.2	16.3 ± 0.2	9.5 ± 0.3	20.5 ± 0.1	16.3 ± 0.3	4.1 ± 0.3[c]	27.2 ± 0.5	16.3 ± 0.3	10.9 ± 0.5
I + LH	26.4 ± 0.2	17.2 ± 0.2	9.2 ± 0.3	22.2 ± 0.1	17.2 ± 0.3	5.0 ± 0.3[ab]	28.8 ± 0.4	17.2 ± 0.3	11.6 ± 0.4
I + 3 E2	25.3 ± 0.2	16.4 ± 0.2	8.9 ± 0.3	21.7 ± 0.1	16.4 ± 0.3	5.3 ± 0.3[a]	27.2 ± 0.4	16.4 ± 0.3	10.8 ± 0.4
I + 300 E2	25.2 ± 0.2	16.5 ± 0.2	8.7 ± 0.3	22.0 ± 0.1	16.9 ± 0.3	5.1 ± 0.3[ab]	27.6 ± 0.5	16.5 ± 0.2	11.1 ± 0.5

[abc]Within an Experiment and a specific IGFBP mRNA, ΔCt values without a common superscript differ ($P < 0.05$).
[d]See Materials and Methods for treatment details and description of Cycle threshold (Ct) method.

Table 3. Quantitative PCR analysis of IGFBP-2, -3 and -4 mRNA expression in large-follicle granulosa (Exp. 4) and theca (Exp. 6) cells treated with no additions (Con), FSH, LH, insulin (I) and(or) estradiol (E2).

	IGFBP-2			IGFBP-3			IGFBP-4		
Treatment[d]	**BP-2 Ct[d]**	**18S Ct**	**ΔCt**	**BP-3 Ct[d]**	**18S Ct**	**ΔCt**	**BP-4 Ct[d]**	**18S Ct**	**ΔCt**
Exp. 4									
Con	27.6 ± 0.3	18.8 ± 0.4	8.8 ± 0.3[a]	24.0 ± 0.2	17.4 ± 0.4	6.6 ± 0.3	28.9 ± 0.6	18.6 ± 0.3	10.4 ± 0.5
I	25.0 ± 0.3	17.9 ± 0.4	7.1 ± 0.3[b]	23.1 ± 0.2	17.4 ± 0.4	5.7 ± 0.3	27.2 ± 0.6	18.0 ± 0.3	9.2 ± 0.4
I + LH	24.9 ± 0.3	17.4 ± 0.4	7.6 ± 0.3[b]	22.6 ± 0.2	16.5 ± 0.3	6.1 ± 0.3	26.4 ± 0.6	17.4 ± 0.3	9.0 ± 0.4
I + FSH	25.1 ± 0.3	17.6 ± 0.4	7.6 ± 0.3[b]	22.2 ± 0.2	16.6 ± 0.3	5.5 ± 0.3	27.1 ± 0.6	17.5 ± 0.2	9.5 ± 0.4
3 E2	24.4 ± 0.3	17.7 ± 0.4	6.7 ± 0.3[b]	22.0 ± 0.2	16.2 ± 0.4	5.8 ± 0.3	26.4 ± 0.6	17.0 ± 0.3	9.4 ± 0.5
300 E2	24.7 ± 0.3	17.7 ± 0.4	7.0 ± 0.3[b]	22.7 ± 0.2	16.7 ± 0.4	6.0 ± 0.3	26.7 ± 0.6	17.7 ± 0.2	9.0 ± 0.4
Exp. 6									
Con	28.6 ± 0.5	18.4 ± 0.3	10.2 ± 0.4	22.9 ± 0.4	18.5 ± 0.2	4.4 ± 0.3[b]	24.6 ± 0.3	18.4 ± 0.3	6.3 ± 0.3[c]
I	28.2 ± 0.4	17.6 ± 0.3	10.7 ± 0.4	22.3 ± 0.4	17.6 ± 0.2	4.7 ± 0.3[b]	24.4 ± 0.3	17.5 ± 0.3	6.9 ± 0.3[b]
E2	29.1 ± 0.4	17.4 ± 0.3	11.6 ± 0.4	23.5 ± 0.4	17.6 ± 0.2	5.9 ± 0.3[a]	26.3 ± 0.3	17.7 ± 0.3	8.5 ± 0.3[a]
E2 + I	28.1 ± 0.4	17.8 ± 0.3	10.3 ± 0.4	23.1 ± 0.4	17.5 ± 0.2	5.7 ± 0.3[a]	27.4 ± 0.3	19.5 ± 0.3	7.9 ± 0.3[ab]
LH	29.3 ± 0.4	18.3 ± 0.3	11.0 ± 0.4	22.9 ± 0.4	18.4 ± 0.2	4.5 ± 0.3[ab]	24.8 ± 0.3	18.5 ± 0.3	6.3 ± 0.3[c]
LH + I	28.3 ± 0.5	17.6 ± 0.3	10.7 ± 0.4	22.3 ± 0.4	17.1 ± 0.3	5.2 ± 0.3[ab]	26.4 ± 0.4	18.9 ± 0.4	7.5 ± 0.3[ab]

[abc]Within an Experiment and a specific IGFBP mRNA, ΔCt values without a common superscript differ ($P < 0.05$).
[d]See Materials and Methods for treatment details and description of Cycle Threshold (Ct) method.

Table 4. Quantitative PCR analysis of IGFBP-2, -3 and -4 mRNA expression in small-follicle (Exp. 3), large-follicle (Exp. 5) granulosa and theca (Exp. 7) cells treated with no additions (Con), IGF-1, IGF-2, FSH and(or) Leptin (LEP).

	IGFBP-2			IGFBP-3			IGFBP-4		
Treatment[f]	BP-2 Ct[f]	18S Ct	ΔCt	BP-3 Ct[f]	18S Ct	ΔCt	BP-4 Ct[f]	18S Ct	ΔCt
Exp. 3									
Con	28.7 ± 0.2	20.1 ± 1.3	8.6 ± 1.4	24.7 ± 0.2	20.1 ± 1.6	4.5 ± 1.7	31.0 ± 0.3	20.1 ± 1.6	10.9 ± 1.7
IGF-1	26.3 ± 0.3	16.6 ± 1.7	9.7 ± 1.8	24.2 ± 0.2	19.2 ± 1.6	4.9 ± 1.7	29.3 ± 0.3	19.2 ± 1.6	10.0 ± 1.7
IGF-2	26.7 ± 0.3	19.5 ± 1.5	7.2 ± 1.6	23.8 ± 0.2	20.5 ± 1.6	3.3 ± 1.7	29.5 ± 0.3	20.5 ± 1.6	9.0 ± 1.7
Exp. 5									
Con	28.6 ± 0.3	19.2 ± 0.4	9.4 ± 0.3[ab]	24.8 ± 0.3	19.2 ± 0.6	5.6 ± 0.5	28.9 ± 0.7	19.1 ± 0.6	9.8 ± 0.5
3 IGF-1	26.9 ± 0.4	18.0 ± 0.4	8.9 ± 0.3[ab]	23.7 ± 0.3	17.7 ± 0.7	6.0 ± 0.6	27.7 ± 0.7	18.1 ± 0.6	9.6 ± 0.5
100 IGF-1	26.2 ± 0.3	17.6 ± 0.4	8.6 ± 0.3[c]	23.3 ± 0.3	17.6 ± 0.5	5.8 ± 0.5	27.0 ± 0.7	17.6 ± 0.6	9.5 ± 0.4
IGF-2	26.5 ± 0.4	17.7 ± 0.4	8.8 ± 0.3[abc]	23.9 ± 0.3	17.9 ± 0.6	6.0 ± 0.5	28.0 ± 0.8	17.8 ± 0.7	10.3 ± 0.5
FSH	27.2 ± 0.4	18.0 ± 0.4	9.8 ± 0.3[a]	24.3 ± 0.3	19.1 ± 0.6	5.1 ± 0.5	28.3 ± 0.7	18.9 ± 0.7	9.3 ± 0.5
LEP	27.6 ± 0.3	18.4 ± 0.4	9.2 ± 0.3[ab]	24.7 ± 0.3	18.9 ± 0.5	5.8 ± 0.5	29.9 ± 0.8	18.5 ± 0.7	11.4 ± 0.5
Exp. 7									
Con	25.9 ± 0.2	21.8 ± 0.4	4.3 ± 0.3[e]	23.1 ± 0.3	21.5 ± 0.4	1.6 ± 0.4	25.6 ± 0.3	21.5 ± 0.4	4.0 ± 0.4
IGF-1	23.7 ± 0.2	19.2 ± 0.3	4.6 ± 0.3[e]	21.3 ± 0.3	19.2 ± 0.3	2.2 ± 0.3	22.8 ± 0.3	19.2 ± 0.3	3.6 ± 0.3
IGF-2	24.5 ± 0.2	18.8 ± 0.4	5.7 ± 0.3[d]	21.3 ± 0.3	18.8 ± 0.4	2.5 ± 0.3	23.3 ± 0.3	18.8 ± 0.4	4.5 ± 0.3

Within an Experiment and a specific IGFBP mRNA, ΔCt values without a common superscript differ [abc]($P < 0.06$) or [de]($P < 0.05$).
[f]See Materials and Methods for treatment details and description of Cycle Threshold (Ct) method.

LITERATURE CITED

ABI Prism Sequence Detection System User Bulletin No. 2. Foster City, CA: PE Applied Biosytems. 11-14.

Adashi, E. Y. 1998. The IGF family and folliculogenesis. J Reprod Immunol 39:13-9.

Armstrong, D. G., G. Baxter, C. G. Gutierrez, C. O. Hogg, A. L. Glazyrin, B. K. Campbell, T. A. Bramley, and R. Webb. 1998. Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinology 139:2146-54.

Armstrong, D. G., C. O. Hogg, B. K. Campbell, and R. Webb. 1996. Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. Biol Reprod 55:1163-71.

Austin, E. J., M. Mihm, A. C. Evans, P. G. Knight, J. L. Ireland, J. J. Ireland, and J. F. Roche. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol Reprod 64:839-48.

Bale, L. K. and C. A. Conover. 1992. Regulation of insulin-like growth factor binding protein-3 messenger ribonucleic acid expression by insulin-like growth factor I. Endocrinology 131:608-14.

Besnard, N., C. Pisselet, D. Monniaux, A. Locatelli, F. Benne, F. Gasser, F. Hatey, and P. Monget. 1996. Expression of messenger ribonucleic acids of insulin-like growth factor binding protein-2, -4, and -5 in the ovine ovary: localization and changes during growth and atresia of antral follicles. Biol Reprod 55:1356-67.

Cataldo, N. A., T. K. Woodruff, and L. C. Giudice. 1993. Regulation of insulin-like growth factor binding protein production by human luteinizing granulosa cells cultured in defined medium. J Clin Endocrinol Metab 76:207-15.

Chamberlain, C. S. and L. J. Spicer. 2001. Hormonal control of ovarian cell production of insulin-like growth factor binding proteins. Mol Cell Endocrinol 182:69-81.

Conover, C. A., G. F. Faessen, K. E. Ilg, Y. A. Chandrasekher, M. Christiansen, M. T. Overgaard, C. Oxvig, and L. C. Giudice. 2001. Pregnancy-associated plasma protein-a is the insulin-like growth factor binding protein-4 protease secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum. Endocrinology 142:2155.

Conover, C. A. and M. C. Kiefer. 1993. Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells. J Clin Endocrinol Metab 76:1153-9.

de la Sota, R. L., F. A. Simmen, T. Diaz, and W. W. Thatcher. 1996. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. Biol Reprod 55:803-12.

Dor, J., N. Costritsci, C. Pariente, J. Rabinovici, S. Mashiach, B. Lunenfeld, H. Kaneti, M. Seppala, R. Koistinen, and A. Karasik. 1992. Insulin-like growth factor-I and follicle-stimulating hormone suppress insulin-like growth factor binding protein-1 secretion by human granulosa-luteal cells. J Clin Endocrinol Metab 75:969-71.

Echternkamp, S. E., H. J. Howard, A. J. Roberts, J. Grizzle, and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. Biol Reprod 51:971-81.

Erickson G.F., D. Ling, S. Shimasaki. N. Ling, S. R. Wietsman and D. A. Magoffin. 1995. Insulin-like growth factor-1 (IGF-1) stimulates IGF binding protein system in rat theca interstitial cells. Endocrine 3: 525-531.

Erickson, G. F., D. Li, R. Sadrkhanloo, X. J. Liu, S. Shimasaki, and N. Ling. 1994. Extrapituitary actions of gonadotropin-releasing hormone: stimulation of insulin-like growth factor-binding protein-4 and atresia. Endocrinology 134:1365-72.

Erickson, G. F., A. Nakatani, N. Ling, and S. Shimasaki. 1992. Cyclic changes in insulin-like growth factor-binding protein-4 messenger ribonucleic acid in the rat ovary. Endocrinology 130:625-36.

Erickson, G. F., A. Nakatani, N. Ling, and S. Shimasaki. 1992. Localization of insulin-like growth factor-binding protein-5 messenger ribonucleic acid in rat ovaries during the estrous cycle. Endocrinology 130:1867-78.

Fielder, P. J., H. Pham, E. Y. Adashi, and R. G. Rosenfeld. 1993. Insulin-like growth factors (IGFs) block FSH-induced proteolysis of IGF- binding protein-5 (BP-5) in cultured rat granulosa cells. Endocrinology 133:415-8.

Fortune, J. E., G. M. Rivera, A. C. Evans, and A. M. Turzillo. 2001. Differentiation of dominant versus subordinate follicles in cattle. Biol Reprod 65:648-54.

Funston, R. N., G. E. Seidel Jr, J. Klindt, and A. J. Roberts. 1996. Insulin-like growth factor I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. Biol Reprod 55:1390-6.

Giudice, L. C., A. A. Milki, D. A. Milkowski, and I. el Danasouri. 1991. Human granulosa contain messenger ribonucleic acids encoding insulin- like growth factor-binding proteins (IGFBPs) and secrete IGFBPs in culture. Fertil Steril 56:475-80.

Greisen, S., A. Flyvbjerg, T. Ledet, and P. Ovesen. 2002. Regulation of insulin-like growth factor binding protein secretion by human granulosa luteal cells in a polycystic ovary-like environment. Fertil Steril 78:162-8.

Grimes, R. W., J. A. Barber, S. Shimasaki, N. Ling, and J. M. Hammond. 1994. Porcine ovarian granulosa cells secrete insulin-like growth factor- binding proteins-4 and -5 and express their messenger ribonucleic acids: regulation by follicle-stimulating hormone and insulin-like growth factor-1. Biol Reprod 50:695-701.

Grimes, R. W. and J. M. Hammond. 1992. Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. Endocrinology 131:553-8.

Grimes, R. W., S. E. Samaras, J. A. Barber, S. Shimasaki, N. Ling, and J. M. Hammond. 1992. Gonadotropin and cAMP modulation of IGF binding protein production in ovarian granulosa cells. Am J Physiol 262:E497-503.

Huynh, H. 2000. Post-transcriptional and post-translational regulation of insulin-like growth factor binding protein-3 and -4 by insulin-like growth factor-I in uterine myometrial cells. Growth Horm IGF Res 10:20-7.

Ingman, W. V., P. C. Owens, and D. T. Armstrong. 2000. Differential regulation by FSH and IGF-I of extracellular matrix IGFBP- 5 in bovine granulosa cells: effect of association with the oocyte. Mol Cell Endocrinol 164:53-8.

Irwin, J. C., B. A. Dsupin, and L. C. Giudice. 1995. Regulation of insulin-like growth factor-binding protein-4 in human endometrial stromal cell cultures: evidence for ligand-induced proteolysis. J Clin Endocrinol Metab 80:619-26.

Iwashita, M., Y. Kudo, and Y. Takeda. 1998. Effect of follicle stimulating hormone and insulin-like growth factors on proteolysis of insulin-like growth factor binding protein-4 in human granulosa cells. Mol Hum Reprod 4:401-5.

Kassem, M., R. Okazaki, D. De Leon, S. A. Harris, J. A. Robinson, T. C. Spelsberg, C. A. Conover, and B. L. Riggs. 1996. Potential mechanism of estrogen-mediated decrease in bone formation: estrogen increases production of inhibitory insulin-like growth factor- binding protein-4. Proc Assoc Am Physicians 108:155-64.

Kim, I., A. Manni, J. Lynch, and J. M. Hammond. 1991. Identification and regulation of insulin-like growth factor binding proteins produced by hormone-dependent and -independent human breast cancer cell lines. Mol Cell Endocrinol 78:71-8.

Kudo, Y., S. Itatsu, M. Iwashita, T. Iguchi, and Y. Takeda. 1995. Effects of estrogen and parathyroid hormone on osteoblastic activity via regulating the binding activity of insulin-like growth factor binding protein-4 in SaOS-2 cells: implications for the pathogenesis of postmenopausal osteoporosis. Biochim Biophys Acta 1245:402-6.

Langhout, D. J., L. J. Spicer, and R. D. Geisert. 1991. Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. J Anim Sci 69:3321-34.

Lapolt, P.S., M. Oikawa, X.C. Jia, and H.J. Hsueh. 1990. Gonadtropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and lutenization. Endocrinology 126:3277-9.

Liu, J., A. T. Koenigsfeld, T. C. Cantley, C. K. Boyd, Y. Kobayashi, and M. C. Lucy. 2000. Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual componentsof the ovarian insulin-like growth factor system. Biol Reprod 63:942-52.

Liu, X. J., M. Malkowski, Y. Guo, G. F. Erickson, S. Shimasaki, and N. Ling. 1993. Development of specific antibodies to rat insulin-like growth factor- binding proteins (IGFBP-2 to -6): analysis of IGFBP production by rat granulosa cells. Endocrinology 132:1176-83.

Martin, J. L., J. A. Coverley, S. T. Pattison, and R. C. Baxter. 1995. Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. Endocrinology 136:1219-26.

Mazerbourg, S., J. Zapf, R.S. Bar, D.R. Brigstock, and P. Monget. 2000. Insulin-like growth factor (IGF)-binding protein-4 proteolytic degradation in bovine, equine and porcine preovulatory follicles: regulation by IGFs and heparin-binding domain-containing peptides. Biol Reprod 63:390-400.

Mazerbourg, S., M. T. Overgaard, C. Oxvig, M. Christiansen, C. A. Conover, I. Laurendeau, M. Vidaud, G. Tosser-Klopp, J. Zapf, and P. Monget. 2001. Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. Endocrinology 142:5243-53.

Mondschein, J. S., S. A. Smith, and J. M. Hammond. 1990. Production of insulin-like growth factor binding proteins (IGFBPs) by porcine granulosa cells: identification of IGFBP-2 and -3 and regulation by hormones and growth factors. Endocrinology 127:2298-306.

Ott, L. In: *An introduction to statistical methods and data analysis*. Duxbury: North Scituate, MA , 215-216. 1977.

Ovesen, P. 1998. Synergistic effects of growth hormone and insulin-like growth factor-I on differentiation and replication of cultured human luteinized granulosa cells. Acta Obstet Gynecol Scand 77:487-91.

Putowski, L., R. M. Rohan, D. S. Choi, W. J. Scherzer, E. Ricciarelli, J. Mordacq, K. E. Mayo, and E. Y. Adashi. 1997. Rat ovarian insulin-like growth factor binding protein-4: a hormone- dependent granulosa cell-derived antigonadotropin. J Soc Gynecol Investig 4:144-51.

Rajaram, S., D. J. Baylink, and S. Mohan. 1997. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18:801-31.

Ricciarelli, E., E. R. Hernandez, A. Hurwitz, E. Kokia, R. G. Rosenfeld, J. Schwander, and E. Y. Adashi. 1991. The ovarian expression of the antigonadotropic insulin-like growth factor binding protein-2 is theca-interstitial cell-selective: evidence for hormonal regulation. Endocrinology 129:2266-8.

Ricciarelli, E., E. R. Hernandez, C. Tedeschi, L. F. Botero, E. Kokia, R. M. Rohan, R. G. Rosenfeld, A. L. Albiston, A. C. Herington, and E. Y. Adashi. 1992. Rat ovarian insulin-like growth factor binding protein-3: a growth hormone-dependent theca-interstitial cell-derived antigonadotropin. Endocrinology 130:3092-4.

Rivera, G. M., and J. E. Fortune. 2001. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. Biol Reprod 65: 112-18.

Rosato, R., K. Gerland, H. Jammes, N. Bataille-Simoneau, B. Segovia, L. Mercier, and A. Groyer. 2001. The IGFBP-3 mRNA and protein levels are IGF-I-dependent and GH- independent in MG-63 human osteosarcoma cells. Mol Cell Endocrinol 175:15-27.

San Roman, G. A. and D. A. Magoffin. 1992. Insulin-like growth factor binding proteins in ovarian follicles from women with polycystic ovarian disease: cellular source and levels in follicular fluid. J Clin Endocrinol Metab 75:1010-6.

SAS . *The SAS system for Windows: Version 8.* Cary, NC: Statistical Analysis Systems Institute. 1999.

Schmid, C., I. Schlapfer, M. Waldvogel, P. J. Meier, J. Schwander, M. Boni-Schnetzler, J. Zapf, and E. R. Froesch. 1992. Differential regulation of insulin-like growth factor binding protein (IGFBP)-2 mRNA in liver and bone cells by insulin and retinoic acid in vitro. FEBS Lett 303:205-9.

Spicer, L. J., P. Alvarez, T. M. Prado, G. L. Morgan, and T. D. Hamilton. 2000. Effects of intraovarian infusion of insulin-like growth factor-I on ovarian follicular function in cattle. Domest Anim Endocrinol 18:265-78.

Spicer, L. J. and C. S. Chamberlain. 2002. Estradiol and luteinizing hormone regulation of insulin-like growth factor binding protein production by bovine granulosa and thecal cells. Endocrine 17:161-8.

Spicer, L. J., C. S. Chamberlain, and G. L. Morgan. 2001. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. Domest Anim Endocrinol 21:1-15.

Spicer, L. J., C. S. Chamberlain, and C. C. Francisco. 2000. Ovarian action of leptin. Endocrin 12:53-59.

Spicer, L. J. and S. E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest Anim Endocrinol 12:223-45.

Spicer, L. J. and C. C. Francisco. 1997. The adipose gene product, leptin: evidence of a direct inhibitory role in ovarian function. Endocrinology 138:3374-78.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, and B. E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J Anim Sci 73:3719-31.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E. Echternkamp. 1996. 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. Endocrinology 137:2842-50.

Voge, J. L., D. T. Allen, J. R. Malayer, and L. J. Spicer. Expression of insulin-like growth-factor binding protein-2, -3, -4 and -5 in fresh versus cultured bovine granulosa and theca cells. Journal of Animal Science 79(Suppl 1), 33. 2001.

Voutilainen, R., S. Franks, H. D. Mason, and H. Martikainen. 1996. Expression of insulin-like growth factor (IGF), IGF-binding protein, and IGF receptor messenger ribonucleic acids in normal and polycystic ovaries. J Clin Endocrinol Metab 81:1003-8.

Wandji, S. A., J. E. Gadsby, F. A. Simmen, J. A. Barber, and J. M. Hammond. 2000. Porcine ovarian cells express messenger ribonucleic acids for the acid- labile subunit and insulin-like growth factor binding protein-3 during follicular and luteal phases of the estrous cycle. Endocrinology 141:2638-47.

Wang, L., X. Ma, L. C. Yeh, and M. L. Adamo. 2001. Differential regulation of IGF-binding protein gene expression by cAMP in rat C6 glioma cells. Endocrinology 142:3917-25.

Yuan, W., B. Bao, H. A. Garverick, R. S. Youngquist, and M. C. Lucy. 1998. Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. Domest Anim Endocrinol 15:55 -63.

CHAPTER IV

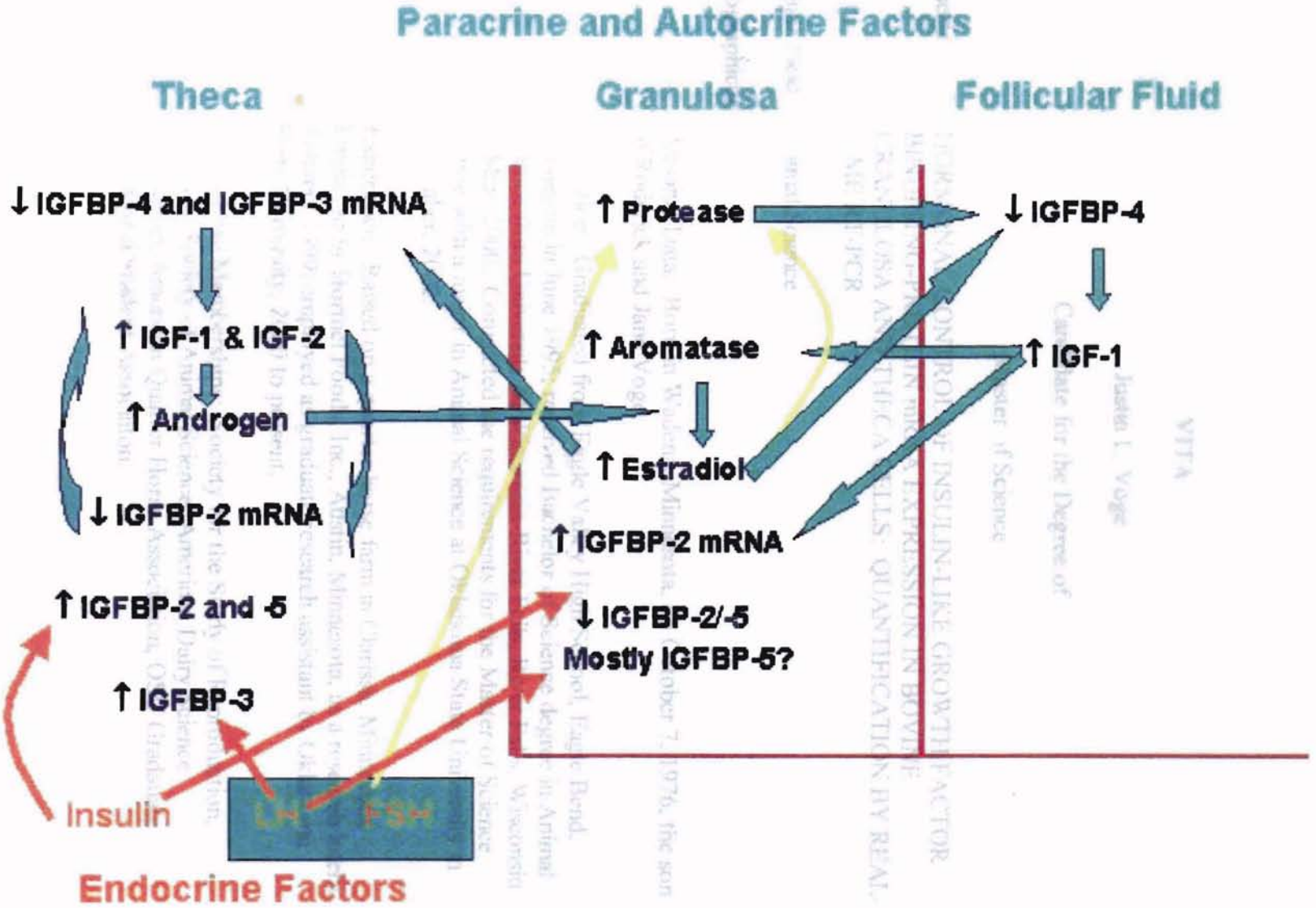
SUMMARY AND CONCLUSIONS

The process of where one follicle is selected to become dominant from a group of follicles is a complex, dynamic process involving a multitude of hormonal factors. Among these factors, IGF-1 is a potent, positive stimulator of ovarian mitogenesis and steroidogenesis. However, IGFBPs, also present within the follicle, bind IGF-1 and regulate (block) its biological activity. Because levels of IGF-1 present in the follicle in cattle remain constant, while levels of IGFBPs fluctuate, changing levels of IGFBPs within dominant growing follicles and subordinate or atretic follicles modulate the stimulatory function of IGF-1. The question still remains unanswered though as to the exact mechanism(s) that manipulate the selection of one follicle to become dominant. However, we postulate a model as illustrated in Figure 10 for the mechanisms that are controlling the ovarian IGFBP system within the dominant follicle.

This study evaluated the effects of several hormones associated with folliculogenesis on the up- or down-regulation of IGFBP-2, -3 or -4 mRNA expression levels in cultured bovine granulosa (small- and large-follicles) and theca (large follicles) cells. IGFBP-2, -3 and -4 mRNA was detected in all cell types examined by fluorescent real-time RT-PCR. Moreover, estradiol, LH, FSH, insulin and IGFs all influenced one or more of the three specific IGFBP mRNA levels measured in the present study. The hormonal effects varied depending on a particular IGFBP's mRNA as well as the cell type examined. How all these hormones interact to control IGFBP mRNA expression

levels and thus synthesis was not completely elucidated. It is likely that throughout the process of follicular development, levels of IGFBP mRNA and therefore protein production are being controlled by a complex network of hormonal events (some of which may be redundant, mutually opposing and (or) synergestic) as well as other factors such as proteases to ultimately control the amount of bioavailable IGF-1 and IGF-2 to the cell. Through a further understanding of the processes that control follicular development and ultimately ovulation of an ovum, hopefully management techniques to improve overall reproductive efficiency of the animal can be developed.

Figure 10. Illustration of a proposed model for the control of the IGFBP system in the dominant bovine ovarian follicle at or around the time of selection. The above model postulates that the first decline seen in IGFBP-4 is more than likely controlled by an IGFBP-4 specific protease. However, what is controlling the activation of this protease has yet to be elucidated (possible controllers are indicated by a yellow arrow). The decline in IGFBP-4 would then lead to an increase in free IGF-1 within follicular fluid of the follicle. The increase in free IGF-1 would increase aromatase activity and thus estradiol output by the granulosa cell layer and at the same time increasing IGFBP-2 mRNA possibly to pull free IGF-1 towards the cell surface. The increase in estradiol would then lead to a decrease in IGFBP-3 and IGFBP-4 mRNA, increasing free IGF-1 as well as IGF-2 in the thecal cell layer. IGF-2 also decreases the amount of IGFBP-2 mRNA produced by the theca layer, all leading to an increase free IGFs. The free IGFs would then increase androgen production by the theca layer, which could then be converted to estradiol by the granulosa cell continuing this cycle. At the same time endocrine factors such as Insulin and LH are increasing IGFBP-2 and -5 as well as IGFBP-3, respectively in the theca cell. This could be to pull free IGFs towards the cell or to increase cell stores of IGF for later use. Furthermore, insulin as well as LH are decreasing IGFBP-2/-5 production by the granulosa cell layer. However, it is possible that this could be predominately IGFBP-5 as IGFBP-2 mRNA was upregulated by insulin and unchanged by LH treatment.


Paracrine and Autocrine Factors
Theca
Granulosa
Follicular Fluid
↓ IGFBP-4 and IGFBP-3 mRNA
↑ IGF-1 & IGF-2
↑ Androgen
↓ IGFBP-2 mRNA
↑ IGFBP-2 and -5
↑ IGFBP-3
↑ Protease
↓ IGFBP-4
↑ Aromatase
↑ IGF-1
↑ Estradiol
↑ IGFBP-2 mRNA
↓ IGFBP-2/-5
Mostly IGFBP-5?
Insulin
LH
FSH
Endocrine Factors

VITA

Justin L. Voge

Candidate for the Degree of

Master of Science

Thesis: HORMONAL CONTROL OF INSULIN-LIKE GROWTH FACTOR BINDINING-PROTEIN mRNA EXPRESSION IN BOVINE GRANULOSA AND THECA CELLS: QUANTIFICATION BY REAL-TIME RT-PCR

Major Field: Animal Science

Biographical:

Personal Data: Born in Wadena, Minnesota, on October 7, 1976, the son of Roderick and Jane Voge.

Education: Graduated from Eagle Valley High School, Eagle Bend, Minnesota in June 1995; received Bachelor of Science degree in Animal Science from University of Wisconsin-River Falls, River Falls, Wisconsin in May, 2000. Completed the requirements for the Master of Science degree with a major in Animal Science at Oklahoma State University in December, 2002.

Experience: Raised on a quarter horse farm in Clarissa, Minnesota. Employed by Hormel Foods, Inc., Austin, Minnesota, as a research intern, Summer 1999; employed as graduate research assistant by Oklahoma State University, 2000 to present.

Professional Memberships: Society for the Study of Reproduction, American Society of Animal Science, American Dairy Science Association, American Quarter Horse Association, OSU Gradauate Professional Student Associaton.