PRODUCTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) BY BOVINE GRANULOSA AND THECAL CELLS: REGULATION BY METABOLIC HORMONES

By

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Bachelor of Science

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Champaign-Urbana, Illinois

1995

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, 1999

PRODUCTION OF INSULIN-LIKE GROWTH FACTOR

BINDING PROTEINS (IGFBPs) BY BOVINE

GRANULOSA AND THECAL CELLS:

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HORMONES

Thesis Approved:

Thesis Adviser

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Leon Spicer. I arrived at Oklahoma State University naïve and unenlightened, looking for my trail through life. Dr. Spicer opened my eyes to the path before me. My resulting journey through the entanglements of study has led me to a wonderful oasis of research. Dr. Spicer has sparked my desire to learn and pushed me to work harder to achieve everything imaginable. I have learned a great deal and will always be indebted.

Dr. Rodney Geisert has been an amazing mentor in which I hold a great deal of respect. He has gone out on shaky limbs for me which are feats I will never forget. His desire to see his students succeed is amazing. I am in awe of his unselfish nature. Thank you, Dr. Geisert for all your help and advice.

I would also like to thank Dr. Robert Wettemann. He is one of the most vivacious researchers I have come to know. He walks into work like a child walks into a toy store. His excitement and compassion for his job is exciting to witness.

Well, I must say, this has been a very fulfilling, adventurous, and at times, difficult experience in which I will always cherish. My duration here has been supplemented with a number of graduate students that I will always remember. If one walks through life, avoiding the special qualities of others', then life has not been lived to the fullest. Thus, I truly want to thank the graduate students that have passed through and currently are in my life. They have listened to my complaints, my humorless jokes

and have made me smile. Thanks.

Finally, I would like to dedicate this thesis to my parents, Wesley and Carolyn Chamberlain. They have sacrificed much for my ambitions and through it all, they have been an amazing frame of support. I am truly proud to be their daughter. Thanks mom and dad.

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CHAPTER I

INTRODUCTION

Poor nutrition is an important limiting factor in the reproductive performance of cattle. When cattle are not sufficiently nourished, they become non-cyclic, consequently resulting in a loss in efficiency and thus profitability. The loss in efficiency is due to a down-regulation of hormonal function. Insulin-like growth factor-I (IGF-I) is one of the hormones adversely affected by reduced nutrient intake. IGF-I stimulates bovine ovarian granulosa and thecal cell growth and differentiation in vitro (Spicer et al., 1993; Spicer and Echternkamp, 1995; Stewart et al., 1995). Bound to IGFs are the high-affinity transport proteins, the insulin-like growth factor binding proteins (IGFBP), which alter bioavailability of IGF. Plasma IGFBP concentrations increase in non-cyclic cattle. Likewise, in 1991, Bicsak et al. found that the IGFBPs inhibited ovulation in rats. Therefore, IGF and the IGFBPs have been proposed to be important regulators of follicular growth and development in cattle (Spicer and Echternkamp, 1995).

At present, little information concerning ovarian IGFBP production is available. Low molecular weight (MW) IGFBPs, IGFBP-2, 29-kDa and 22-kDa IGFBPs, are negatively correlated with follicular fluid concentrations of IGF-I in cattle (Echternkamp et al., 1994). Thus, growing antral follicles have decreased

concentrations of the low MW IGFBPs, making IGF-I more bioavailable. Conversely, atretic follicles have increased concentrations of the low MW IGFBPs making IGF-I less bioavailable. In contrast, IGFBP-3 is involved in maintaining intrafollicular IGF-I concentrations (Echternkamp et al., 1994). Because IGFBP production is influenced not only by normal follicular development, but by factors such as poor nutrition, studies focused on these proteins may find new methods for improved fertility. Thus, the objective of this study was to determine the hormonal regulation of IGFBP production by bovine granulosa and thecal cells, with specific focus on the effects of insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), cortisol, and glucagon on IGFBP production.

CHAPTER II

REVIEW OF LITERATURE

OVARIAN INSULIN-LIKE GROWTH FACTORS (IGFs)

The insulin-like growth factors (IGF-I and -II) are single chain polypeptides consisting of an intact C-peptide and three intramolecular disulfide bonds, which are structurally similar to proinsulin. The amino acid sequence composing IGF-I and -II are highly homologous across species. For example, the amino acid sequence in sheep, pigs, humans, and rats are 99%, 100%, 100%, and 96% homologous to bovine IGF-I, respectively (for review see, Spicer and Echternkamp, 1995). Likewise bovine IGF-II is 99%, 97%, 96% and 96% homologous to the ovine, porcine, human and rat, respectively (for review see, Spicer and Echternkamp, 1995). These highly conserved growth factors are present in many tissue fluids and peripheral circulation (Rosenfeld et al., 1996).

The activity of IGF-I is modulated by high affinity type-I IGF receptors (Nissely et al., 1985). The type-I receptor also binds IGF-II and insulin, but only with 10% and .3% cross-reactivity, respectively (Baranao and Hammond, 1984; Davoren et al., 1986; Gates et al., 1987; Adashi et al., 1988b; Sauerwein et al., 1992). Like insulin, IGF-I acts through the tyrosine kinase pathway which links to the mitogen-activated protein kinase signal transduction system and transduces the cellular signal to the nucleus. The IGF-II receptor consists of a single glycosylated protein chain, approximately 250 kDa. This

IGF type II receptor is identical to the mannose-6-phosphate receptor (Kiess et al., 1994). These receptors have been localized within the mammalian ovary.

The IGFs have been identified in a variety of tissues. Within the ovary, IGF-I mRNA has been detected in cultured rat (Adashi et al., 1989; Oliver et al., 1989), porcine (Hatey et al., 1992), and bovine (Spicer and Echternkamp, 1995) granulosa cells, ovine CL (Einspanier et al., 1990; Perks et al., 1995) and bovine thecal cells (Spicer et al., 1993). Gene expression for IGF-I has not been detected in human (Voutilainen and Miller, 1987; El-Roeiy et al., 1993; Geisthovel et al., 1989; Hernandez et al., 1992) granulosa cells or ovine follicles (Perks et al., 1995; Spicer et al., 1995). However, gene expression for IGF-II is present in human stromal, thecal, and granulosa cells (Voutilainen et al., 1996) and ovine (Perks et al., 1995) thecal cells. Further observations have indicated that the greatest gene expression of IGF-II is detected in the human granulosa cells of the large follicle with expression increasing during follicular growth, likely due to the stimulatory effect of the gonadotropins (Voutilainen et al., 1996). Similar to IGF-II mRNA levels in human granulosa cells, concentrations of IGF-I increase with follicular growth in cattle (Spicer et al., 1988; Spicer and Enright, 1991) and pigs (Bryan et al., 1989; Spicer et al., 1992). Likewise the IGF-I receptor is located in granulosa cells of rats (Oliver et al., 1989) and cattle (Spicer et al., 1994; Stewart et al., 1996), and in pigs (Otani et al., 1985; Caubo and Tonetta, 1989), cattle (Stewart et al., 1995; 1996) and mice (Hernandez et al., 1988a,b; Cara and Rosenfield, 1988) thecal cells. The IGF-I gene is expressed in human thecal cells (Bergh et al., 1993; El-Roeiy et al., 1993; 1994).

Components of the IGF axis play an important role in cellular proliferation and follicular development. Within the ovary, IGFs increase cell proliferation in cultures of human (Erickson et al., 1990; Olsson et al., 1990), ovine (Monniaux and Pisselet, 1992), bovine (Savion et al., 1981; Spicer et al., 1993), rat (Adashi et al., 1985) and porcine (Baranao and Hammond, 1984; Veldhuis and Furlanetto, 1985; Maruo et al., 1988; Kamada et al., 1992) granulosa cells and bovine thecal cells (Stewart et al., 1995). IGFs also act synergistically with gonadotropins to stimulate aromatase activity (Adashi et al., 1985; Kamada et al., 1992) and progesterone biosynthesis (Savion et al., 1981; Adashi et al., 1985; Monniaux and Pisselet, 1992), as well as to regulate formation of LH/hCG receptors (Adashi et al., 1985; Davoren et al., 1985; 1986) in granulosa cell cultures. Likewise, within cultured thecal and secondary interstitial cells, IGF-I and LH synergistically stimulate androgen production (Magoffin and Erickson, 1988; Cara and Rosenfeld, 1988; Magoffin et al., 1990; Hernandez et al., 1988b). Hormones regulate IGF-I receptors. EGF, estradiol, and FSH increased and bFGF decreased the number of IGF-I receptors in granulosa cells of small bovine follicles (Spicer et al., 1994). Similarly, LH had no effect, whereas bFGF decreased the number of IGF-I receptors in thecal cells of large bovine follicles (Spicer and Stewart, 1996). In the same experiment, LH or progesterone had no effect on the concentration of IGF-receptors (Spicer et al., 1994). Hormones such as estradiol (Veldhuis et al., 1986) and dexamethasone (Urban et al., 1994) also increase IGF-I receptor numbers in porcine granulosa cells.

Another factor involved in the IGF axis is the insulin-like growth factor binding proteins. Each IGFBP preferentially binds to IGF-I and(or) -II at different affinities to regulate the transport and bioavailability of the IGFs (Table 1). Currently six IGFBPs

have been cloned and sequenced with each IGFBP exhibiting high homology across species (Table 2). Estradiol concentrations are negatively correlated with IGFBP-2, the 27-29-kDa IGFBP (presumably IGFBP-5), and 24-kDa IGFBP (presumably IGFBP-4) in ovine and bovine follicular fluid, whereas IGFBP-3 is not (Spicer et al., 1995; Stewart et al., 1996). IGFBP-2, -3, -4, and -5 attenuate IGF-I- and FSH-induced estradiol synthesis in cultured rat, bovine, and human granulosa cells by sequestering IGF-I (Ui et al., 1989; Mason et al., 1992; Liu et al., 1993; Spicer and Chamberlain, 1999). Thus, throughout the estrous cycle IGF/IGFBPs are continually modified with the changing endocrine profiles, demonstrating the complexity of the IGF axis.

OVARIAN INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs)

Chemistry, biological function and ovarian function

IGFBP-1

IGFBP-1 is a nonglycosylated, acid stable protein (Ooi and Herington, 1988; Baxter and Martin, 1989a) with a molecular weight of 30-31 kDa (Shimasaki and Ling, 1991) that binds IGF-II with greater affinity than IGF-I (Table 1). The protein sequence of IGFBP-1 shares 68-72% homology among species (Table 2) and contains 12 N-terminal and 6-C terminal cysteine residues, both of which are required for optimal IGF-I and -II binding (Lee et al., 1997). IGFBP-1 is synthesized primarily in the liver (Lee et al., 1993) and is present in amniotic fluid (Giudice et al., 1991) as well as plasma (Rabinovici et al., 1997) of humans. IGFBP-1 is also present in a number of human ovarian components such as preovulatory follicular fluid, corpora lutea (CL; Seppala et

al., 1984), as well as luteal cells of hyperstimulated preovulatory follicles (Seppala et al., 1984), and cultured thecal (Mason et al., 1991), stromal (Mason et al., 1991) and luteinized granulosa cells (Suikkari et al., 1989; Jalkanen et al., 1989; Giudice et al., 1991). Human IGFBP-1 mRNA, but not the protein, was only expressed in cultured thecal cells whereas both the protein (Jalkanen et al., 1989) and mRNA (El-Roeiy et al., 1994) for IGFBP-1 were detected in granulosa cells. Moreover, concentrations of IGFBP-1 are greater in follicular fluid than serum (Hartshorne et al., 1990).

IGFBP-1, along with IGF-I and -II, is involved in a complex system that regulates the menstrual cycle, ovulation, decidualization, blastocyst implantation, and fetal growth (for review see, Lee et al., 1993; Lee et al., 1997), working possibly through the adenylate-cyclase and protein-kinase-C-dependent pathways (Jalkanen et al., 1989). Within the ovary, IGFBP-1 is associated with luteinized granulosa cells of the CL, which suggests IGFBP-1 is involved with luteinization of the granulosa cells (Jalkanen et al., 1989; Suikkari et al., 1989; Giudice et al., 1990a; Hartshorne et al., 1990) and regulation of the CL (Van Dessel et al., 1996). During the preovulatory stage, IGFBP-1 concentrations in serum (Martikainen et al., 1991) and follicular fluid (Van Dessel et al., 1996) are significantly increased, suggesting that IGFBP-1 may regulate follicular growth and maturation possibly by mediating the actions of IGF-I. Thecal cell androgen production is controlled by IGF-I in synergy with LH. Thus, it is possible that during the follicular phase, IGFBP-1 inhibits IGF-I-induced androgen production by thecal cells and thus prevents follicular atresia (Nobels and Dewailly, 1992). However, due to post-translational modifications, the actions of IGFBP-1 are not clear. IGFBP-1 can both inhibit and stimulate IGF-binding to cell surface receptors,

thereby controlling IGF-mediated mitogenic and metabolic actions in various tissues, including the ovary (Holst et al., 1997). The inhibitory form of IGFBP-1 is phosphorylated. This phosphorylation of IGFBP-1 causes a six-fold increase in its affinity for IGF-I (Jones et al., 1991). Therefore, when the affinity of IGFBP-1 for IGF-I is substantially higher than that of the receptor, the equilibrium will favor the binding of IGF-I by the binding proteins in preference to binding of its receptor (Clemmons, 1993). Conversely, dephosphorylated IGFBP-1 has a lower affinity for IGF-I, thus allowing more interaction of IGF-I with its receptor (Clemmons, 1993).

IGFBP-2

IGFBP-2, which binds to both IGF-I and -II with high affinity (Roghani et at., 1991), is a nonglycosylated, acid stable (Ooi and Herington 1988; Baxter and Martin, 1989a) protein with a molecular mass of approximately 29-40 kDa. The amino acid sequence of IGFBP-2 exhibits 64-92% homology among species (Table 2). This is the major binding protein in cerebrospinal fluid and the central nervous system (Binoux et al., 1982; Baxter et al., 1989a; Rosenfeld et al., 1989), human ovary (Voutilainen et al., 1996), and is also present in adult rat serum (Shimasaki et al., 1991b), human milk and seminal plasma (Binoux et al., 1991a,b; Blum et al., 1993). IGFBP-2 gene expression is also localized in the bovine endometrium, and to a lesser extent, myometrium (Kirby et al., 1996). In general, IGFBP-2 binds IGF-II with greater affinity than IGF-I (Table 1) and appears to inhibit IGF action, primarily IGF-II (Reeve et al., 1993). In bovine thecal cells, IGFBP-2 inhibits IGF-I-induced cell proliferation and steroidogenesis (Spicer et al., 1997). However, within certain cell-types, IGFBP-2 does have a modest stimulatory influence on IGF action (Bar et al., 1989).

IGFBP-2 is abundant in ovarian tissues of all species examined, but its cellular localization varies among species (Zhou et al., 1996). Within the ovary, IGFBP-2 mRNA has been localized in the granulosa and thecal cells and CL of pigs (Samaras et al., 1992) and sheep (Perks and Wathes, 1996), ovine stromal (Perks and Wathes, 1996) and the thecal-interstitial and secondary interstitial cells of the rat (Nakatani et al., 1991). In humans, IGFBP-2 protein (Cataldo et al., 1993; Mason et al., 1996) and mRNA (Voutilainen et al., 1996) has been detected in the stromal, granulosa and thecal cells. Bovine IGFBP-2 protein (Funston et al., 1996) is also located in the granulosa and the thecal cells but gene expression has only been detected in the CL (Kirby et al., 1996) and granulosa cells (Yuan et al., 1998). Specifically, bovine IGFBP-2 mRNA was present in granulosa cells of the early and mid-dominant follicle (Yuan et al., 1998) although the protein level appeared to decrease in dominant follicles (Stewart et al., 1996). In the case of the human, IGFBP-2 mRNA is expressed more in the thecal tissue than in the granulosa cells or stromal tissue of the large (14 mm) follicle (Voutilainen et al., 1996). The reverse is true in the small (11 mm) human follicle where IGFBP-2 mRNA is higher in the granulosa cells than the larger follicle or thecal compartment (Voutilainen et al., 1996). It appears that IGFBP-2 production decreases as granulosa cells differentiate and luteinize, and aromatase activity increases. This and other research suggests IGFBP-2 behaves in an autocrine manner to regulate individual follicle response to trophic factors (Samaras et al., 1992).

In human (Cwyfan Hughes et al., 1997) and bovine (Stanko et al., 1994) follicular fluid, IGFBP-2 is denoted by three bands, two of which correspond to non-IGF-binding proteolytic fragments. In the human, the smallest band (23-kDa), detected

by immunoblotting, represents the major form of this binding protein in the fluid of dominant follicles (Cwyfan Hughes et al., 1997). In contrast, only intact IGFBP-2 (34-kDa) is located in conditioned medium from human granulosa cells, thecal, and stromal explants (Cwyfan Hughes et al., 1997). However, both the intact (34-kDa) and a 25.5-kDa band are seen in the circulation of women (Cwyfan-Hughes et al., 1997). The discovery of lower molecular weight forms of IGFBP-2 in follicular fluid suggests a specific mechanism for modulating IGFBP-2, since the 23-kDa is not found in any other compartment of the human ovary, nor is it the same as that found in the circulation. It is possible that the 23-kDa band is a result of proteolysis of IGFBP-2, which also has been demonstrated in ovine follicular fluid (Besnard et al., 1996b). Proteolysis of the IGFBPs is discussed in more detail in a later section of this review.

IGFBP-3

IGFBP-3 is a N-glycosylated (Zapf et al., 1988) 150 kDa binding protein consisting of an acid-labile 85 kDa subunit, an IGF peptide, and a binding subunit (Baxter and Martin 1989b; Rosenfeld et al., 1990; Lamson et al., 1991) which exists in two alternatively glycosylated forms with molecular weights of approximately 44- and 42-kDa. These two glycoprotein forms decrease in size to a single species upon deglycosylation (Liu et al., 1990), the amino acid sequence of which is highly conserved among species (Table 2). Absence of the carbohydrate side chains may not permit IGFBP-3 to associate with cell surfaces (McCusker et al., 1990). Immunoblots revealed both the intact doublet (43- and 40-kDa) and a lower molecular weight band (29-kDa) were present in medium conditioned by thecal extracts as well as atretic follicles in the human (Cwyfan-Hughes et al., 1997). Of the six IGFBPs, approximately 75-80% of

liver-derived IGFBP-3 binds to IGFs in the circulation (Guler et al., 1989; Zapf et al., 1990; Baxter, 1994), but not to insulin (Clemmons et al., 1986). Binding affinity of IGFBP-3 for IGF-I and -II are similar (Table 1). Thus, this ternary complex acts as a circulating reservoir of IGFs and regulates the delivery of the IGFs to target tissues. The half-life (i.e., metabolic clearance) of IGF-I bound to this complex is 3-6 h whereas free IGF-I has a half life of 20-30 min (Zapf et al., 1986; Davis et al., 1989; Guler et al., 1989; Hodgkinson et al., 1989; Bassett et al., 1990). Also by sequestering IGF-I and preventing interaction with its receptor, IGFBP-3 can inhibit IGF-I bioactivity. DeMellow and Baxter (1988) demonstrated that when cultured human fibroblasts are treated simultaneously with IGF-I and IGFBP-3, IGFBP-3 inhibits IGF-I-stimulated proliferation in a dose-dependent manner. Furthermore, when cultured human fibroblasts are first treated with IGFBP-3 for 24 h, followed by IGF-I, IGF-I-induced cell proliferation increased (DeMellow and Baxter, 1988). An inhibitory action of IGFBP-3 on IGF-I-induced steroidogenesis and proliferation of bovine granulosa (Spicer and Chamberlain, 1999) and thecal (Spicer et al., 1997) cells has been confirmed. IGFBP-3 also inhibits gonadotropin-induced steroid production by rat (Ui et al., 1989) and human (Mason et al., 1992; San Roman and Magoffin, 1993) granulosa cells. Thus, ovarian IGFBP-3, which has been shown to neutralize gonadotropin and IGF-I action, likely works by its ability to sequester IGFs (Baranao and Hammond, 1984; Hammond et al., 1988; Kamada et al., 1992; Spicer et al., 1997). Conversely, an increase in IGF-I action has been attributed to cell surface-associated IGFBP-3 (Conover, 1992). For example, in vitro work has shown that in fetal human fibroblasts and porcine smooth muscle cells, IGFBP-3 enhances IGF-I binding to receptors (McCusker et al., 1991a).

IGFBP-3 has been located in a variety of tissues, including the liver, testis, and ovary. Moreover, IGFBP-3 gene expression has been detected in the bovine endometrium (Kirby et al., 1996). Within the ovarian compartment, IGFBP-3 gene expression is not detected in the rat granulosa or thecal cells, but the IGFBP-3 mRNA has been found in the CL (Nakatani et al., 1991) undergoing regression (Erickson et al., 1993a). Similar localization of ovarian porcine IGFBP-3 mRNA (Mondschein et al., 1990; Samaras et al., 1992) comply with the rat except that low levels of IGFBP-3 mRNA were detected in thecal cells (Samaras et al., 1994). Contrary to the rat, human IGFBP-3 mRNA was detected in cultured granulosa cells (Giudice et al., 1991) and was induced in cultured thecal cells (El-Roeiy et al., 1994; Voutilainen et al., 1996). A modest amount of IGFBP-3 mRNA was also detected in bovine granulosa cells whereas none was found in the thecal cells from small antral follicles (Yuan et al., 1998). Furthermore, IGFBP-3 protein has been localized in both human (Cataldo et al., 1993) and porcine (Mondschein et al., 1990) granulosa cells, as well as in the human thecal and stromal tissue (Hughes et al., 1997). The concentration of IGFBP-3 in follicular fluid is constant throughout the estrous cycle as demonstrated in cows (Echternkamp et al., 1994a; de la Sota, et al., 1996; Funston et al., 1996; Stewart et al., 1996), sheep (Monget et al., 1993; Spicer et al., 1995), and pigs (Grimes et al., 1994b). It is also constant during the human menstrual cycle (Cataldo and Giudice, 1992b; Huang et al., 1994). However, San Roman and Magoffin (1993) have found that as human follicles reach the estrogenic ovulatory phase, IGFBP-3 concentrations decrease. Moreover, IGFBP-3 follicular levels are comparable to those seen in serum (Cataldo et al., 1992b; Stanko et al., 1994; Stewart et al., 1996). This indicates that intrafollicular IGFBP-3

originates from an endocrine source. As mentioned, the liver is the main site of gene expression and secretion of IGFBP-3, which then forms large complexes in the serum (Wood et al., 1988). Prior to ovulation, vascularization of the follicle is increased (Okuda et al., 1983) which would potentiate the passage of IGFBP-3 from serum into follicular fluid. It is therefore possible that follicular fluid IGFBP-3 is derived from the peripheral circulation, although, some of IGFBP-3 may originate from ovarian cells (Cataldo et al., 1992b). Furthermore, luteal IGFBP-3 may act in a paracrine manner to limit follicular growth during the luteal phase (Samaras et al., 1992).

IGFBP-4

The identity of IGFBP-4 can not be resolved conclusively, but it appears as a 22-24-kDa nonglycosylated binding protein that contains one potential asparagine-linked glycosylation site (Shimasaki et al., 1990) and binds IGF-I and IGF-II with equal affinity (Table 1). IGFBP-4 also consists of an approximately 28-kDa glycosylated protein (Fielder et al., 1990), the amino acid sequence of which is highly conserved among species (Table 2). IGFBP-4 was originally isolated from the conditioned medium of human bone cells and rat serum (LaTour et al., 1990; Shimasaki et al., 1990) and has since been identified in body fluids (i.e., seminal plasma), and conditioned media of several cell lines, including normal human osteoblast-like cells (Mohan et al., 1989), fibroblasts (Camacho-Hubner et al., 1992), endometrial stromal cells (Irwin et al., 1995) and neuroblastoma cells (Fielder et al., 1990; Cheung et al., 1991).

Human IGFBP-4 is primarily localized in granulosa cells of atretic follicles, (Cataldo et al., 1993; Voutilainen et al., 1996) and its mRNA is expressed in increasing levels during atresia (Nakatani et al., 1991; Erickson et al., 1992a; Erickson et al.,

1992b). IGFBP-4 has also been detected in the CL and oocyte at the primordial stage of follicular growth and persists in the preantral and antral stage of follicular development (Peng et al., 1996). Using human thecal-conditioned medium from dominant follicles, Cwyfan-Hughes et al. (1997) demonstrated the presence of the non-glycosylated IGFBP-4 (24-kDa) along with two possible fragments of IGFBP-4 (16.5- and 15-kDa) which are not visible by ligand blotting. Moreover, a glycosylated intact IGFBP-4 (26-kDa) was found in human follicular fluid (Cwyfan-Hughes et al., 1997). Human IGFBP-4 mRNA is also moderately expressed in the thecal, stromal, and granulosa cells of large (14 mm) preantral follicles, but is 50% more abundant in granulosa cells of smaller follicles (11 mm; Voutilainen et al., 1996). Interestingly, immunostaining revealed that in human antral follicles from polycystic ovaries without hyperinsulinemia, IGFBP-4 is more prominent in granulosa cells as compared to thecal cells during the early follicular phase (Peng et al., 1996). However, IGFBP-4 is more prominent in thecal cells compared to granulosa cells in women suffering from polycystic ovarian syndrome with hyperinsulinemia during the early follicular phase (Peng et al., 1996).

Similar to the human, IGFBP-4 is prominent in rat granulosa cells of atretic follicles (Liu et al., 1993; Choi et al., 1996). However, rat IGFBP-4 mRNA was not detected in oocytes, thecal interstitial cells, or the theca externa (Erickson et al., 1992a) but a weak and variable signal for mRNA has been detected in dominant follicles (Erickson et al., 1992a). IGFBP-4 mRNA is present in ovine thecal cells, and to an extent, granulosa cells of healthy follicles, with no significant differences between small and large follicles (Besnard et al., 1996a). However, expression of ovine IGFBP-4 mRNA is greater in thecal cells from small late atretic, compared with small normal and

small atretic follicles (Besnard et al., 1996a). In comparison, IGFBP-4 is detected more in atretic and small healthy follicles compared to large healthy follicles in pigs (Besnard et al., 1997). However, Zhou et al. (1996) reported that IGFBP-4 mRNA was not present in small follicles of pigs. Rather, IGFBP-4 mRNA in pigs first appears in thecal cells of medium sized growing follicles, and does not appear in the granulosa cells of the dominant follicles until the late follicular phase, when luteinization is evident (Gadsby et al., 1996; Zhou et al., 1996). IGFBP-4 mRNA is also present in the porcine CL (Zhou et al., 1996).

Follicular fluid from large estrogen-inactive, medium and small bovine follicles contain detectable levels of IGFBP-4 (Funston et al., 1996). Stewart et al. (1996) found a decrease in IGFBP-4 activity in early dominant follicles when compared with subordinate small and large follicles of cattle. Moreover, the percentage of follicular fluid to plasma IGFBP-4 activity in small follicles resulted in a 131% difference, suggesting ovarian production of IGFBP-4 (Stewart et al., 1996). Because IGFBP-4 is significantly increased in normal progesterone/androgen-dominant follicular fluid compared with estrogen-dominant follicular fluid (Cataldo and Giudice, 1992a; Cataldo and Giudice, 1992b), gonadotropin-dependent maturation of follicles may be due to inhibition of the expression and virtual disappearance of granulosa cell-derived IGFBP-4. Consequently, high IGFBP-4, like other IGFBPs, may be an inhibitor of IGF-I action in small and large estrogen-inactive follicles. Similar to other IGFBPs, IGFBP-4 binds to IGF thereby preventing the interaction of the IGFs with their receptors. By inhibiting IGF action in the ovary, IGFBP-4 may inhibit follicular development and induce atresia. For instance, IGFBP-4 inhibits IGF-I-stimulated estradiol release by rat (Liu et al., 1993)

and human (Iwashita et al., 1996) granulosa cells in vitro. Likewise, addition of IGFBP-4 to cultured rat granulosa cells in the presence of either 100 or 30 ng/ml of FSH inhibited estradiol production (Ui et al., 1989; Liu et al., 1993). However, because a decrease in intrafollicular IGFBP-4 levels during follicular growth is not associated with a decrease in expression in the corresponding mRNA, the absence of IGFBP-4 protein in follicular fluid from the preovulatory follicles may be attributed to proteolytic degradation (see later section on IGFBP proteolysis; Fowlkes et al., 1995).

IGFBP-5

IGFBP-5 is a 25 amino acid O-linked glycosylated (Conover and Kiefer, 1993) binding protein with a molecular weight of approximately 29-31 kDa (Shimasaki and Ling, 1991; Shimasaki et al., 1991b). The amino acid sequence of IGFBP-5 is highly homologous among mammals (Table 2). Like the other IGFBPs, IGFBP-5 contains 18 conserved cysteines (Jones and Clemmons, 1995) which may be involved in specific binding to the IGFs, with IGF-II having a greater affinity than IGF-I for IGFBP-5 (Table 1). A soluble form of IGFBP-5 has no Asn-linked glycosylation sites and does not contain the Arg-Gly-Asp which is believed to facilitate binding to cell surface integrin receptors (Ruoslahti et al., 1987). However, IGFBP-5 also consists of a sequence of 13 highly basic amino acids near the carboxyl terminus which may facilitate binding to the extracellular matrix and decrease its affinity for IGF-I (Clemmons, 1993). This 21-kDa extracellular matrix-bound IGFBP is the more predominant form of IGFBP-5 (Clemmons, 1993).

IGFBP-5 mRNA has been identified in the rat liver, brain, lung, heart, spleen, stomach, kidney, adrenal, intestine and testis homogenates (Erickson et al., 1992b), as

well as in human fibroblasts (Camacho-Hubner et al., 1992) and osteoblast (Conover and Kiefer 1993; Canalis and Gabbitas, 1995) cells. Within the rat ovary, IGFBP-5 mRNA is expressed in atretic granulosa cells, secondary interstitial cells, CL, and surface epithelium (Erickson et al., 1992b). Erickson and co-workers (1992b) found that rat IGFBP-5 mRNA is constantly expressed in the surface epithelium. During proestrus, the message to IGFBP-5 is present in granulosa cells of a few atretic preantral follicles. Around the onset of estrus, the IGFBP-5 gene expression is very prominent in almost every atretic preantral follicle. In addition, gene expression for IGFBP-5 is detected in granulosa cells of some atretic antral (graafian) follicles and secondary interstitial cells. Finally, during diestrus I and II, IGFBP-5 mRNA is decreased and essentially absent in all follicles (healthy and atretic) and the secondary interstitial cells. No evidence of IGFBP-5 mRNA was found in rat granulosa cells of dominant follicles, oocytes, thecal interstitial cells, thecal externa, stromal or smooth muscle cells regardless of the stage of the estrous cycle (Erickson et al., 1992b). However, using an antibody specific to rat IGFBP-5, Liu et al. (1993) demonstrated that rat cultured granulosa cells produce IGFBP-5. Thus, these results reveal tissue-specific expression of the rat IGFBP-5 gene as well as stage (and perhaps hormone) dependent regulation of ovarian IGFBP-5 mRNA. In humans, IGFBP-5 mRNA has been localized within the ovarian compartment. Within the granulosa (Zhou et al., 1993; Voutilainen et al., 1996) and thecal cells (Voutilainen et al., 1996) IGFBP-5 mRNA has been detected, but is primarily localized in the ovarian stroma (Zhou et al., 1993).

A 30-kDa IGFBP in pigs has tentatively been identified as IGFBP-5. Amounts of IGFBP-5 are greater in porcine small healthy follicles than large healthy follicles, and in

atretic compared to healthy follicles, whatever the follicle size (Besnard et al., 1997). In addition, the granulosa cells have been found to secrete IGFBP-5 (Grimes et al., 1994a). Although not abundant, ovarian IGFBP-5 mRNA is also present in the surface epithelium, blood vessels, luteal capillaries, connective tissue and vasculature of pig ovaries (Zhou et al., 1996). IGFBP-5 mRNA, however, has not been found in porcine follicles (Zhou et al., 1996). This evidence suggests that IGFBP-5 is not critical in follicular selection or function, at least in the pig.

Similar to the pig, a 30-kDa IGFBP produced by large and small granulosa cells, in vitro, has a suggested identity of IGFBP-5 in sheep (Armstrong et al., 1996). Ovine IGFBP-5 mRNA is primarily present in the granulosa cells of atretic follicles, and the thecal cells of healthy follicles (Besnard et al., 1996a). In follicular fluid, IGFBP-5 levels, detected by ligand blotting, are greater in small versus large ovine follicles (Spicer et al., 1995). In follicular fluid, IGFBP-5 levels, detected by ligand blotting, are greater in small versus large ovine follicles (Spicer et al., 1995). Therefore, atresia of large follicles is associated with an increase in intrafollicular IGFBP-5 levels and an increase in IGFBP-5 mRNA expression in granulosa cells and a slight decrease in IGFBP-5 mRNA expression in thecal cells (Besnard et al., 1996a).

IGFBP-5 has also been tentatively identified in cattle, using immunoblotting techniques, as a doublet in follicular fluid with a molecular range of 29-31-kDa (Funston et al., 1996). In addition, Stanko et al. (1994) reported a 21-kDa fragment in bovine follicular fluid that corresponds to IGFBP-5. Stewart et al. (1996) who also reported a single band representing IGFBP-5 (29-kDa), found that during the first follicular wave of the bovine estrous cycle, IGFBP-5 activity was greater in subordinate small (<6 mm in

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diameter) and large (≥ 6 mm in diameter) follicles compared with early dominant follicles (Stewart et al. 1996) which has been confirmed by others (Carolan et al., 1996; de la Sota et al., 1996).

Due to proteolysis, the intact IGFBP-5 is degraded to smaller molecular weight fragments of approximately 16-20-kDa. However, the association of IGFs may stimulate the increase in IGFBP-5. Binding of IGF to IGFBP-5 protects it from proteolysis by FSH-treated granulosa cells (Fielder et al., 1993). Proteolysis of IGFBP-5 is discussed in detail in a later section. Moreover, the addition of IGFBP-5 to cultured rat granulosa cells in the presence of either 100 or 30 ng/ml of FSH inhibited estradiol production (Liu et al., 1993). These studies imply that during follicular growth, IGFBP-5 production is maintained or increases when bound to IGF while FSH levels are increasing. However, the effect of IGFBP-5 on IGF action remains contradictory. Kiefer et al. (1992) demonstrated that rhIGFBP-5 inhibited IGF-stimulated DNA synthesis in human osteoblastic cells. In contrast, Andress and Birnbaum (1991) found that partially purified IGFBP-5 enhanced IGF-stimulated osteoblast mitogenesis. These conflicting results could be due to the nature of IGFBP-5. Kiefer et al. (1992) utilized intact IGFBP-5 whereas Andress and Birnbaum (1991) used a 23-kDa form of IGFBP-5. This 23-kDa protein may just represent a proteolytic fragment that is functionally distinct from IGFBP-5. IGFBP-5 fragments have a decreased affinity for IGF and therefore increases IGF bioavailability. In addition, when IGFBP-5 is associated with the extracellular matrix in fibroblasts and bone (Bautista et al., 1991; Jones et al., 1992), it has a decreased affinity for the IGF peptides and stimulates IGF action. Therefore, these

studies infer that soluble IGFBP-5 may inhibit, whereas matrix-associated IGFBP-5 may enhance IGF action (Conover and Kiefer, 1993).

IGFBP-6

IGFBP-6 is an approximately 28-32-kDa binding protein found primarily in humans and rats. IGFBP-6, which binds IGF-II with the highest affinity of the binding proteins (Table 1), also binds IGF-I except with a 20-100 fold lower affinity (Forbes et al., 1990; Martin et al., 1990). IGFBP-6 has been purified from various tissues, including human cerebrospinal fluid (Bach et al., 1992; Bach et al., 1994), serum (Zapf et al., 1990), media conditioned by transformed fibroblasts (Martin et al., 1990), keratinocytes (Kato et al., 1995), prostate cancer cells (Srinivasan et al., 1996), osteoblasts (Gabbitas and Canalis, 1997), bovine mammary epithelial cells (Cohick and Turner, 1998) and retinal pigment epithelial cells (Feldman and Randolph, 1994). Within the ovary, IGFBP-6 gene has been localized in the rat theca-interstitial compartment (Rohan et al, 1993) and appears to be produced by rat granulosa cells. Using RT-PCR, human IGFBP-6 mRNA is found in the thecal, stromal, and granulosa cells (Voutilainen et al., 1996). In addition, Shimasaki et al. (1990) found IGFBP-6 in porcine follicular fluid. However, it is not found in porcine granulosa cells, thus suggesting that IGFBP-6 is produced by other ovarian cells in the pig.

The structure of IGFBP-6 is not phosphorylated, unlike IGFBP-1, -3, and -5, which may be serine-phosphorylated. Furthermore, IGFBP-6 lacks 2 and 4 of the N-terminal cysteines in the human and rat, respectively, that are normally conserved in IGFBP-1 through -5 (Shimasaki et al., 1991a) and are believed to be involved with IGF-binding (Spencer and Chan, 1995). In addition, IGFBP-6 is an O-glycosylated protein.

Human IGFBP-6 contains an additional N-glycosylation area, but the site is not glycosylated. As previously mentioned, glycosylation of the binding proteins not only serve for ligand binding, but also facilitate cellular processing, protection from proteolysis, modulation of binding to structural biomolecules including membrane and extracellular matrix components and retardation of in vivo clearance. Therefore, glycosylation of IGFBP-6 may prolong its half-life, due to decreased clearance by specific receptors and also, protect the protein from proteolysis. However, deglycosylation of human IGFBP-6 has no effect on IGF binding, so it would appear that glycosylation has no role in IGF-II binding by IGFBP-6 (Neumann et al., 1998). Unlike the other IGFBPs, rhIGFBP-6 has no effect on FSH-induced steroid production by rat granulosa cells (Rohan et al., 1993).

Hormonal Regulation of IGFBP-1

Growth Factors

IGF. IGFs are produced within the ovarian follicle, stimulate mitogenesis and steroidogenesis, and regulate IGFBP activity. Addition of IGF-I or -II to human granulosa-luteal cells reduces IGFBP-1 levels significantly (Dor et al., 1992; Poretsky et al., 1996). The potency of IGF-I and -II were similar but much greater than that of insulin (Poretsky et al., 1996). Interestingly, IGFBP-1 inhibits IGF-I and -II-stimulated bovine granulosa cell division (Lavranos et al., 1996). To add to the complexity, IGF-I treatment increased IGFBP-1 concentrations by 6-8-fold in cultured fibroblast cells (Hill et al., 1989) but decreased secretion in cultured rat hepatocytes (Scharf et al., 1996) and human endometrial cells (Clemmons et al., 1990; Thrailkill et al., 1990). These results

suggest that the specific effect of IGF-I and -II on IGFBP-1 production may be cell type dependent as previously suggested (Camacho-Hubner et al., 1991).

EGF. Within the ovarian compartment, EGF enhances the mitogenic activity of IGF-I in cultured granulosa (Savion et al., 1981; May et al., 1988; Vernon and Spicer, 1994) and thecal cells (May et al., 1992; Spicer and Stewart, 1996) but inhibits steroidogenesis (Langhout et al., 1991; Spicer and Stewart, 1996). In pigs and humans, as follicle size increases, levels of EGF-like activity decrease (Hsu et al., 1987; Westergaard et al., 1990). From this work, it is postulated that EGF may be involved with enhancing growth of small antral follicles as well as slowing the terminal maturation of preovulatory follicles (Monniaux et al., 1997). The action of EGF on IGFBP-1 production remains in question, however. EGF increases IGFBP-1 secretion in human granulosa-luteal cells (Angervo et al., 1992; Yap et al., 1998), as well as human hepatoma cells (Angervo, 1992) and endometrial cancer cells (Angervo et al., 1991). Similarly, when pigs are administered EGF for 4 weeks, serum IGFBP-1 levels are increased (Vinter-Jensen et al., 1996). In contrast, 50 ng/ml and 10 ng/ml EGF had no effect or reduced IGFBP-1 concentrations in cultured human intestinal epithelial cells (Oguchi et al., 1993) and porcine aortic smooth muscle cells (Clemmons and Gardner, 1990), respectively. These results suggest that as with IGF-I, the specific effect of EGF on IGFBP-1 production is dependent on cell-type. From the ovarian studies, one could imply that the combination of IGFBP-1 and EGF may regulate follicular growth to inhibit follicular atresia.

bFGF. Within the bovine ovary, bFGF is produced by the CL (Gospodarowicz et al., 1985; 1986; Stirling et al., 1990; 1991) and granulosa cells (Neufeld et al., 1987)

while its receptors are found on ruminant thecal and granulosa cells (Wandji et al., 1992; DiBlasio et al., 1993; Schams et al., 1993). In addition, bFGF receptors and mRNA have been identified in bovine luteal cells (Stirling et al., 1990, 1991) and human granulosa cells (DiBlasio et al., 1993). Like EGF, bFGF enhances granulosa cell proliferation, but inhibits steroidogenesis in many mammals, including cattle (Gospodarowicz and Bialecki, 1978, 1979; Gospodarowicz et al., 1985;Wandji et al., 1992; Vernon and Spicer, 1994). Specifically, bFGF inhibits progesterone production in pig (Channing et al., 1983; Biswas et al., 1988), rat (Yamoto et al., 1993) and bovine thecal cells (Spicer and Stewart, 1996) and FSH-induced estrogen production in rat (Baird and Hsueh, 1986; Adashi et al., 1988a; Yamoto et al., 1993) and bovine granulosa cells (Vernon and Spicer, 1994).

Few studies have detailed the effects of bFGF on the ovarian IGFBPs. In cultured human granulosa cells, bFGF did not consistently alter IGFBP-1 secretion (Yap et al., 1998). In porcine aortic smooth muscle cells, bFGF does not compete with IGF-I for binding to IGFBP-1 (Clemmons and Gardner, 1990). Because bFGF is localized within the ovarian compartments and is involved in inhibiting steroidogenesis, as previously discussed, bFGF may play a permissive role to enhance growth and delay terminal maturation of follicles (Monniaux et al., 1997).

Gonadotropins

FSH. In humans, developing, preovulatory follicles progressively accumulate FSH whereas atretic follicles diminish in FSH concentrations (McNatty et al., 1975). In cattle, a rise in systemic FSH preceeds a wave of follicular growth and selection of a dominant follicle (Ginther et al., 1996). Thus, follicles either die by atresia or are

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rescued by high concentrations of FSH. Down- regulation of the IGFBPs, possibly caused by high concentrations of FSH, permits IGF-I and FSH to synergize and promote follicle growth and development. Conseqently, IGFBPs are generally not detected in large estrogen-active follicles prior to the LH surge in cattle, excluding IGFBP-3 (Echternkamp et al., 1994a). In humans (Cataldo and Giudice, 1992a,b; Giudice et al., 1993; San Roman and Magoffin, 1993), rats (Erickson et al., 1992a,b), and pigs (Grimes et al., 1994a; Howard and Ford, 1992), IGFBP-2, -4, and -5 have antigonadotropic effects on ovarian stimulation by FSH. FSH, acting through the cAMP pathway, may decrease IGFBP release and enhance the access of endogenously generated IGFs to their cognate cell surface receptors and hence, cellular hormonal action. However, the effect of FSH on IGFBP-1 production does not conclusively support the antigonadotropic potential proposed. Mason et al. (1993) demonstrated that 0.1-10 ng/ml FSH increased IGFBP-1 concentration by cultured human granulosa cells primarily from large (> 5 mm) follicles (Mason et al., 1993). Another study which contradicted Mason et al. (1993), demonstrated FSH (Jalkanen et al., 1989; Ovesen et al., 1994) or the combination of GH and FSH did not affect IGFBP-1 production of human granulosa cells (Ovesen et al., 1994). However, the experiment did not include large preovulatory follicles, like the study by Mason et al. (1993). Finally, Adashi et al. (1991) concluded that FSH affects, in a biphasic manner, IGFBP-1 production by rat granulosa cells. Low doses of FSH stimulated IGFBP-1 whereas higher doses inhibited IGFBP-1 production in vitro (Adashi et al., 1990a; Adashi et al., 1991). The low dose of FSH, used by Mason agreed with these studies. Thus, these different results may be explained, in part, by the different size of follicles used within each experiment or the different concentrations of FSH applied.

LH. Pulsatile LH secretion plays an important role in follicular development in cattle (Spicer and Echternkamp, 1986; Ginther et al., 1996), and acquisition of LH receptors in granulosa cells is a critical step in follicular development (Spicer and Echterkamp, 1986; Ginther et al., 1996; Stewart et al., 1996; Bao et al., 1997). How LH and the IGF system regulate each other is still unclear. Stewart et al. (1995) demonstrated that IGF-I increases LH/hCG binding sites in bovine thecal cells, and similar results are seen in rat granulosa cells (Adashi et al., 1985; Davoren et al., 1985; 1986). However, LH (10 ng/ml) or hCG (100 ng/ml) had no effect on IGFBP-1 mRNA or its protein by human thecal (Voutilainen et al., 1996) or granulosa-luteal cells cultured 48 h (Jalkanen et al., 1989), respectively. Conversely, Giudice et al. (1991) demonstrated that IGFBP-1 increased 6 to 8-fold with the addition of 10 ng/ml of hCG to human luteinized granulosa cells cultured for 72 h. Higher doses (100 and 1000 ng/ml) of hCG did not as effectively impact IGFBP-1 production (Giudice et al., 1991). Studies involving uterine stromal tissue have confirmed the stimulatory effect of hCG on IGFBP-1 production (Tang et al., 1993; Han et al., 1995). These studies suggest IGFBP-1 production by granulosa cells is dependent on the dose and duration of LH/hCG treatment, and further substantiate an interactive association between LH and the ovarian IGF system.

Steroids

Estradiol. Levels of circulating estradiol increase progressively with the growth of the follicle and the increase in thecal and granulosa cell numbers. Once the critical estradiol level is attained, it activates the release of GnRH and consequently, LH. Thus, the increase in estradiol is crucial for folliculogenis and ovulation. An increase in low

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molecular weight IGFBPs in bovine follicular fluid is associated with a loss of estrogenic capacity by large non-ovulatory follicles before the preovulatory surge of LH (Echternkamp et al., 1994a; Roberts and Echternkamp, 1995). Subsequent changes in preovulatory follicular steroidogenesis following the preovulatory surge of LH may be independent of IGF-I and/or IGFBP regulation, but loss of estrogenic capacity by dominant non-ovulatory follicles in cattle is associated with increases in IGFBP-2, -4, and 5 levels (Funston et al., 1996; Stewart et al., 1996). Estradiol inhibits IGFBP-1 protein and mRNA levels by human luteinizing granulosa cell cultures (Iwashita et al., 1994) and rat endometrial tissue (Molnar and Murphy, 1994), respectively. However, in vivo work suggests that estrogen stimulates serum (Martikainen et al., 1992) and endometrial (Fazleabas et al., 1989) IGFBP-1 concentrations in human and non-human primates, respectively. These studies suggest that IGFBP-1 levels are regulated differently between species and cell-type.

Cortisol. Glucocorticoids inhibit follicular function by inhibiting FSH-induced aromatase activity in cultured rat granulosa cells (Hsueh and Erickson, 1978). Likewise, glucocorticoids can inhibit somatic growth in humans by inhibiting GH and IGF-I activity (Miell et al., 1993; 1994). However, in the cow, cortisol increases IGF-I-induced granulosa and thecal cell numbers, and increase progesterone and androstenedione production in thecal cells (Spicer and Chamberlain, 1998). Hence, there is evidence that glucocorticoids may be involved directly in folliculogenesis. Glucocorticoids may have an essential role in hepatic IGFBP-1 expression (Conover et al., 1993a; Lee et al., 1993). In vivo, dexamethasone decreased serum IGFBP-1 in healthy human males (Miell et al., 1993; 1994). However, dexamethasone increased plasma IGFBP-1 levels in rats (Luo et

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al., 1990) and humans (Conover et al., 1993a). In vitro work using rat (Orlowski et al., 1990) and human (Powell et al., 1993) hepatoma cells supports the stimulatory action of dexamethasone on IGFBP-1 production. Reasons for the varied results among studies are uncertain. Whether cortisol or other glucocorticoids affect ovarian IGFBP-1 production remains to be determined.

Metabolic Hormones

GH. GH receptors or its mRNA have been demonstrated in human (Tamura et al., 1994), bovine (Tanner and Hauser, 1989), and rat (Carlsson et al., 1993) granulosa cells. In the presence of insulin, GH stimulates granulosa cell growth in the cattle (Langhout et al., 1991) and swine (Hsu and Hammond, 1987). Likewise, GH stimulates steroidogenesis in cultured rat (Jia et al., 1986), human (Ovesen et al., 1994), and porcine (Hsu and Hammond, 1987; Samaras et al., 1994) granulosa cells, and bovine thecal cells (Spicer and Stewart, 1996). GH treatment alone or in combination with FSH or estradiol stimulates IGF-I production by cultured porcine (Hsu and Hammond, 1987). GH may also indirectly alter ovarian IGF action by modulating intrafollicular levels of the IGFBPs (for review see, Rechler, 1993). Indeed, at 10 or 100 ng/ml in vivo and 0.1 mg/kg in vitro, GH increased IGFBP-1 levels in human follicular fluid (Rabinovici et al., 1997) and spent culture medium from granulosa cells (Ovensen et al., 1994), respectively. In contrast, GH (10 ng/ml) had no effect on cultured human thecal cell IGFBP-1 mRNA production (Voutilainen et al., 1996). This lack of effect corresponds with the finding that human IGFBP-1 mRNA was only found to be expressed in cultured thecal cells not the thecal cell, per se. Similar to the thecal cells, no GH effect was observed in cultured rat hepatocytes (Scharf et al., 1996). Upon injection of or

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immunization against GH, in postmenopausal women and (Kassem et al., 1998) mice (Palmer et al.1994), respectively, serum IGFBP-1 levels decreased. Moreover, upon co-administration of bGH and the antiserum to GH, IGFBP-1 serum levels were comparable to the control group (Palmer et al., 1994). Thus, most studies support the stimulatory action of GH on IGFBP-1 protein levels.

Insulin. Insulin stimulates granulosa cell estradiol (Veldhuis and Furlanetto, 1985; Poretsky and Kalin, 1987; Maruo et al., 1988; Caubo et al., 1989; Urban et al., 1990; Spicer and Chamberlain, 1998) and thecal androgen (Caubo et al., 1989; Morley et al., 1989; Stewart and Spicer, 1995) production in several species. Pigs, exogenously administered insulin increases ovulation rate (Cox et al., 1987) and decreases follicular atresia (Matamoros et al., 1990; 1991) whereas insulin increases follicular fluid estradiol concentrations in superovulated cattle (Simpson et al., 1994). Edwards et al. (1996) research demonstrated that absence of insulin reduced follicular growth and IGF-I levels in the acute (6 days) diabetic pig. Thus, exogenous insulin positively affects intrafollicular IGF-I (Meurer et al., 1991; Cox et al., 1994; Simpson et al., 1994). The absence of insulin during diabetes mellitus also altered the IGFBPs in cultured porcine follicles in the presence of androgen substrate without affecting steroidogenesis (Edwards et al., 1996). Serum levels of insulin in healthy and diabetic subjects (Suikkari et al., 1988; Lee et al., 1993), as well as streptozocin-induced diabeteic pigs (Edwards et al., 1996) are inversely related to IGFBP-1 concentrations. Insulin exerts an inhibitory influence on IGFBP-1 production at the transcriptional level (Orlowski et al., 1991; Powell et al., 1991). Insulin also enhances the turnover rate of IGFBP-1 by stimulating its mobility through the endothelium wall (Bar et al., 1990a,b). In vitro, 25 or 250 ng/ml

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of insulin and 2 mg/ml of insulin-conditioned medium inhibited production of IGFBP-1 protein (Holst et al., 1997) and mRNA (Cataldo et al., 1993), respectively, by human granulosa cells. However, 1 or 10 ng/ml of insulin, which are within the physiologic range (Diamond et al., 1985), did not significantly reduce IGFBP-1 production in cultured human luteinized granulosa cells (Poretsky et al., 1996). Scharf et al. (1996) demonstrated that insulin decreased IGFBP-1 concentrations in a dose-dependent manner by cultured rat hepatocytes.

Glucagon. In contrast to insulin, glucagon stimulates glucose output within the liver. Insulin primarily inhibits IGFBP secretion. Because of the opposing effect between glucagon and insulin, one could speculate that IGFBP secretion would elevate in the presence of glucagon. Research involving human fetal liver explants (Lewitt and Baxter, 1989), and cultured rat liver cells (Kachra et al., 1991; Denver and Nicoll, 1994) support this concept. In one such study, healthy and GH deficient patients experienced an increase in serum IGFBP-1 when administered glucagon treatments (Hilding et al., 1993). Serum insulin and glucose were also increased, but decreased subsequent to the IGFBP-1 increase (Hilding et al., 1993). The decrease by insulin would imply that at physiological levels, glucagon has an overriding stimulatory effect thereby negating the domination usually induced by insulin. This is further supported by the increases observed with both IGFBP-1 and insulin after food consumption (Gibson et al., 1995). Moreover, phosphorylation of IGFBP-1 is not changed with glucagon treatments. As previously stated, IGFBP-1 is highly phosphorylated upon secretion from the liver (Westwood et al., 1994). However, non- or less-phosphorylated forms of IGFBP-1 levels are also evident as demonstrated in the maternal circulation during pregnancy

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(Westwood et al., 1994). This suggests hepatic posttranslational processing of IGFBP-1 is unaffected by hormonal treatments (Westwood et al., 1995). In spite of the numerous reports that confirm the increase in IGFBP-1 with glucagon treatment, there are cases that are in contrast with these studies. Others found no stimulating effect by glucagon on IGFBP-1 concentrations, as exemplified by cultured human hepatoma cells (Powell et al., 1991) and primary rat hepatocytes (Villafuerte et al., 1992). Jensen et al. (1991) did not find a consistent response by serum IGFBP-1 concentrations with glucagon administration. Reasons for the discrepancies among studies may be due to differences in treatment conditions as well as cell types and species.

Hormonal Regulation of IGFBP-2

Growth Factors

IGF. IGF-II (30 ng/ml) inhibited IGFBP-2 production by 49% compared to the controls in cultured human granulosa cells (Cataldo et al., 1993). In addition, 30 ng/ml of [Leu^{27}]IGF-II, which binds to type II but not type I IGF receptors, (Beukers et al., 1991) decreased IGFBP-2 to 29% of controls in cultured human granulosa cells (Cataldo et al., 1993). However, 30 ng/ml IGF-I did not affect IGFBP-2 production (Cataldo et al., 1993). These results indicate that ovarian IGFBP-2 production may be mediated through the IGF type II receptor (Cataldo et al., 1993). However, few effects by the type II receptor have been reported for IGFBP-2 in other cell types. In cultured vascular smooth muscle cells, IGFBP-2 secretion remains relatively unchanged with IGF-I or -II treatments (Bourner et al., 1992; Cohick et al., 1993), and IGF-I inhibited IGFBP-2 production by cultured rat hepatocytes (Scharf et al., 1996).

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EGF. IGFBP-2 production was not effected by EGF in cultured porcine granulosa cells (Mondschein et al., 1990), human intestinal epithelial cells (Oguchi et al., 1993) or vascular smooth muscle cells (Bourner et al., 1992; Cohick and Clemmons, 1993). However, IGFBP-2 mRNA production increased with the addition of EGF to cultured rabbit proximal tubular cells (Yap et al., 1997). These results indicate EGF is ineffective on influencing IGFBP-2 protein levels, but may influence IGFBP-2 transcription by some cell types.

bFGF. IGFBP-2 production was not influenced by bFGF in cultured vascular smooth muscle cells (Bourner et al., 1992; Cohick and Clemmons, 1993; Cohick et al., 1995). However, bFGF increased IGFBP-2 in a dose-dependent manner by human cultured neuroblastoma cells (Babajko et al., 1997).

Gonadotropins

FSH. IGFBP-2 release is inhibited by FSH (200 ng/ml) in cultured porcine (Mondschein et al., 1990; Grimes et al., 1992) and rat (Bicsak et al., 1990) granulosa cells. However, treatment with 100 ng/ml of FSH had no significant effect on IGFBP-2 levels in human luteinizing granulosa cells (Cataldo et al., 1993). These discrepancies may stem from the different FSH concentrations utilized.

LH. hCG treatment of weaned sows from 0-18 h decreased porcine follicular fluid IGFBP-2 (Howard and Ford, 1992). In addition, from 18 to 24 h, follicular IGFBP-2 levels remained low and then dramatically increased at 30-36 h post-hCG (Howard and Ford, 1992). Therefore, it was not until sows approached ovulation that IGFBP-2 levels began to increase (Howard and Ford, 1992). In contrast, LH inhibits IGFBP-2 production by human granulosa cells (Giudice et al., 1991; Cataldo et al.,

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1993) and ovine thecal cells (Armstrong et al., 1996). LH (10 ng/ml) had no effect on IGFBP-2 mRNA production by cultured human thecal cells (Voutilainen et al., 1996). When 100 ng/ml of hCG was applied for 2 days to cultured human luteinizing granulosa cells, IGFBP-2 levels decreased with hCG after 2 days; after 5 days the decrease was more marked (Cataldo et al., 1993). Another experiment was set up to determine that hCG was, in fact, inhibiting synthesis of IGFBP-2 (Cataldo et al., 1993). Cultured human granulosa cells were first treated with or without hCG for 2 days. After 2 days the cells were then treated with or without cycloheximide, a protein synthesis inhibitor, in the absence of hCG (Cataldo et al., 1993). IGFBP-2 levels which were increased by cyclohexamide treatment was likely due to the release of intracellular stores of IGFBP-2 (Cataldo et al., 1993). Cells treated with hCG and then cycloheximide released less IGFBP-2 compared to untreated cells (Cataldo et al., 1993). This suggests that hCG attenuates releasable intracellular stores and the hCG-stimulated decrease in IGFBP-2 release does not result from increased intracellular accumulation of IGFBP-2 (Cataldo et al., 1993).

Steroids

Estradiol. Estradiol is stimulatory to rat theca-interstitial cell IGFBP-2 mRNA (Ricciarelli et al., 1991) whereas, synthesis of IGFBP-2 protein is inhibited by estradiol in rat and pig granulosa cells (Adashi et al., 1990; Mondschein et al., 1990; Riciarelli et al., 1991). In normal healthy humans (San Roman and Magoffin, 1993), bovine (Echternkamp et al., 1994a; Funston et al., 1996; Stewart et al., 1996), ovine (Spicer et al., 1995), and porcine (Howard and Ford, 1992) follicular fluid, IGFBP-2 tended to decrease with increasing estradiol concentrations. Moreover, IGFBP-2 in follicular fluid,

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as demonstrated in the pig, may be associated with differentiation during the transition from follicular to luteal tissue (Howard and Ford, 1992).

Cortisol. Dexamethasone and hydrocortisone stimulate secretion of IGFBP-2 protein and mRNA by cultured mouse pancreatic β-cells (Katz et al., 1997) and rabbit proximal tubular cells (Yap et al., 1997), respectively. However, dexamethasone inhibited IGFBP-2 production in cultured porcine stromal vascular cells (Chen et al., 1995). Whether cortisol affects IGFBP-2 production by granulosa or thecal cells remains to be determined.

Metabolic Hormones

GH. GH may affect amount of IGFBP-2 in serum but not in follicular fluid Administration of GH to prepurbertal gilts for 20 and 40 days decreased IGFBP-2 in serum whereas follicular fluid IGFBP-2 activity was not affected (Samaras et al., 1994). Likewise, humans injected with 0.1 mg/kg GH, had no significant effect on IGFBP-2 in follicular fluid (Rabinovici et al., 1997). However in humans (Hardouin et al., 1989; Clemmons et al., 1991; Blum et al., 1993) and transgenic mice (Camacho-Hubner et al., 1991) deficient in GH, serum IGFBP-2 levels were elevated, though short-term changes in GH did not affect circulating levels of IGFBP-2 (Jorgensen et al., 1993). GH of cattle was similar to the human and mouse where IGFBP-2 serum levels decreased and IGF-I increased (Cohick and Clemmons, 1993; Stanko et al., 1994), but gene expression of IGFBP-2 was not effected in the ovary (Kirby et al., 1996). In heifers immunized against GH-releasing hormone, which decreases serum GH and IGF-I, serum and follicular fluid IGFBP-2 levels are increased (Cohick et al., 1996). IGFBP-2 production by human luteinizing granulosa (Cataldo et al., 1993) and IGFBP-2 mRNA in thecal cells

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(Voutilainen et al., 1996) and rat hepatocytes (Scharf et al., 1996) were not influenced by GH treatment in vitro. These results indicate that GH treatment in vivo or in vitro, has no effect on ovarian IGFBP-2 production.

Insulin. Although insulin stimulates IGFBP-2 production in cultured porcine granulosa cells (Grimes and Hammond, 1992; Samaras et al., 1993) the effects of insulin appeared to depend on insulin concentration. For example, if rat intestinal epithelial cells are cultured with low doses of insulin (1.0-5.0 μg/ml), IGFBP-2 levels increased whereas high doses (10 μg/ml) decreased IGFBP-2 levels (Guo et al., 1995). Similar results were also reported in cultured rat hepatocytes (Scharf et al., 1996). Although the lower dose (5 μg/ml) increased IGFBP-2 protein levels in cultured rat intestinal epithelial cells, IGFBP-2 mRNA was decreased (Guo et al., 1995). The increase in IGFBP-2 secretion therefore appears to be at a translational or posttranslational level whereas the decrease in IGFBP-2 may be due to the dramatic reduction in IGFBP-2 mRNA expression (Guo et al., 1995). Human serum IGFBP-2 levels remained unchanged during an overnight fast or after ingestion of a morning meal (Clemmons et al., 1991). These data indicate that circulating IGFBP-2 is not altered by acute fluctuations in insulin at physiological doses. However, Edwards et al. (1996) demonstrated an increase in follicular fluid IGFBP-2 levels in diabetes mellitus-induced pigs. Moreover, when human subjects are fasted for 9 days, IGFBP-2 increases 1.7-fold (Clemmons et al., 1991). So IGFBP-2 may be elevated in cases of extreme and/or long-term insulin deficiency, such as insulin-dependent diabetes mellitus. IGFBP-2 levels, which can cross the endothelial barrier (Bar et al., 1990a,b), may serve as a stable intermediate level of control in IGF action between IGFBP-3 which does not cross the endothelial barrier and IGFBP-1, which undergoes

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acute, metabolically induced fluctuations. Therefore, chronic changes in insulin secretion affect serum IGFBP-2, although adaptation to insulin is relatively slow (Bereket et al., 1995).

Glucagon. Few, if any studies have analyzed the effects of glucagon on ovarian IGFBP-2 concentrations. However, Denver and Nicoll (1994) investigated whether glucagon effected IGFBP-2 levels by cultured rat hepatocytes. Although IGFBP-1 levels were increased, IGFBP-2 activity was not influenced by glucagon treatment (Denver and Nicoll, 1994). Whether glucagon alters ovarian IGFBP-2 production remains to be elucidated.

Hormonal Regulation of IGFBP-3

Growth Factors

IGF. IGF treatment has rather consistent effects on IGFBP-3 production among the various cell types. IGF-I, IGF-II and [Leu^{27}]IGF-II all increased IGFBP-3 in cultured human luteinized granulosa cells supplemented with insulin (Cataldo et al., 1993), with insulin having no effect. Grimes and Hammond (1992) reported similar IGF treatment effects on IGFBP-3 production by porcine granulosa cells. Outside the ovary, IGF-I treatment increased IGFBP-3 mRNA by two- (Camacho-Hubner et al., 1992), and 6-fold (Bale and Conover, 1992) in cultured human fibroblasts and bovine fibroblasts, respectively, but had no effect on cultured human retinal pigment epithelial cells (Feldman and Randolph, 1994). The increase in mRNA in human fibroblasts is similar to the observed increase in IGFBP-3 protein level (Camacho-Hubner et al., 1992). IGFBP-3 production was also increased in cultured human keratinocytes, but its message

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decreased subsequent to IGF-I treatment (Wraight and Werther, 1995). The increase in IGFBP-3, due to IGF-I, or -II, appears to be due to enhanced synthesis of IGFBP-3 (Camacho-Hubner et al., 1992). Moreover, because IGFBP-3 has a greater affinity for IGF-I than the IGF-I receptor (McCusker et al., 1990), the IGF peptides may act to stimulate preformed IGFBP-3 through a nonreceptor-mediated mechanism, possibly by interaction with cell surface-adherent IGFBP-3 (Cataldo et al., 1993). Release of IGFBP-3 in bovine (McCusker et al., 1990) and human (Conover, 1991; Neely and Rosenfeld, 1992) cultured fibroblasts have in fact been shown to be non-receptor mediated.

EGF. The effect of EGF on IGFBP-3 production is inconsistent across experiments and cell type. Within the ovary, EGF did not effect IGFBP-3 production levels by cultured porcine granulosa cells (Mondschein et al., 1990). Likewise, EGF did not significantly affect IGFBP-3 protein or mRNA by cultured human intestinal- (Oguchi et al., 1993) or retinal pigment-epithelial cells (Feldman and Randolph, 1994), respectively. In contrast, EGF increases IGFBP-3 synthesis by cultured rat astroblasts (Loret et al., 1991) and human retinal pigment epithelial cells (Feldman and Randolph, 1994). Moreover, yet another cell line exhibited contradictory responses to the above cell types. EGF treatments decreased IGFBP-3 protein and mRNA levels by cultured human cervical epithelial cells (Andreatta-Van Leyen et al., 1994) and keratinocytes (Wraight and Werther, 1995) and mouse embryo fibroblasts (Villaudy et al., 1991). Vinter-Jensen et al. (1996) likewise reported a decrease in serum IGFBP-3 levels in goettingen minipigs administered EGF daily for 4 weeks. The conglomeration of results would suggest, the IGFBP-3 response to EGF appears to vary with cell type/sample.

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bFGF. IGFBP-3 levels from cultured rat astroblasts decreased in response to bFGF (Loret et al., 1991). Like other hormone interactions previously discussed, the variable results are likely due to the differences in cell types.

Gonadotropins

FSH. FSH is generally inhibitory or without effect on ovarian IGFBP-3 levels. For example, FSH inhibited IGFBP-3 production by cultured human granulosa cells from polycystic ovarian follicles (San Roman and Magoffin, 1992) as well as porcine granulosa cells (Mondschein et al., 1990; Grimes et al., 1992). Likewise, administration of FSH to hypophysectomized rats, resulted in decreased IGFBP-3 mRNA in theca-interstitial cells (Rohan et al., 1993). However, IGFBP-3 levels from large follicles of cyclic cattle were not influenced by exogenous administration of FSH (Echternkamp et al., 1994a). Data from these experiments suggest the difference between species impact the interaction between FSH and IGFBP-3.

LH. IGFBP-3 levels among preovulatory and nonovulatory follicles remained relatively constant before and after the LH surge in cattle (Funston et al., 1996). Likewise, levels of serum IGFBP-3 are similar to follicular levels in cattle (Echternkamp et al., 1994a; Funston et al., 1996). However, ligand blots revealed porcine IGFBP-3 in blood and follicular fluid decreased in a linear manner from 0 to 36 h after in vivo treatments of 500 IU hCG (Howard and Ford, 1992). In vitro work in cultured human thecal (Voutilainen et al., 1996) and granulosa cells (Giudice et al., 1991) indicated LH and hCG had no effect on IGFBP-3 mRNA, but decreased IGFBP-3 protein levels in granulosa cells (San Roman and Magoffin, 1992). Therefore, the influence of LH on IGFBP-3 appears to be determined by type of cell and species.

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Steroids

Estradiol. Although the low molecular weight IGFBPs in follicular fluid are negatively correlated with estradiol concentration, IGFBP-3 levels are not effected. Estrogen enhances IGFBP-3 mRNA in a dose-dependent manner in primary rat osteoblast cultures (Schmid et al., 1989), but has no effect on IGFBP-3 production by porcine granulosa cells (Mondschein et al., 1990). Likewise, estradiol did not effect IGFBP-3 protein serum levels of infertile women undergoing in vitro fertilization (Salobir et al., 1996) or in cultured human fibroblasts (Camacho-Hubner et al., 1992). However, exogenous administration of estradiol to cattle resulted in an increase in IGFBP-3 serum levels. These discrepancies may be due to a variety of influences such as cell type, species differences, and variation in treatment.

Cortisol. Like many of the hormone treatments previously discussed, the influence of cortisol on IGFBP-3 production by various cell types is inconsistent. Miell et al. (1993; 1994) showed that dexamethasone in vivo increased IGF-I and IGFBP-3 levels in healthy males. In contrast, treatment of cultured human fibroblasts with dexamethasone decreased IGFBP-3 production (Camacho-Hubner et al., 1992) but had no effect on human keratinocytes (Wraight and Werther, 1995). IGFBP-3 mRNA levels were likewise affected, exhibiting an inhibitory response to glucocorticoid treatment in human osteoblast-like cells (Okazaki et al. 1994). Thus a possible post-translational mechanism may affect IGFBP-3 production in osteoblast-like cells. However, due to the differences in cell culture systems, the reason for IGFBP-3 regulation by cortisol is inconclusive. Thus, whether cortisol affects ovarian IGFBP-3 is unknown.

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Metabolic Hormones

GH. Somatotropin appears to play an influential role in regulating circulating IGFBP-3. Somatotropin treatments to lactating cows (Cohick et al. 1993) and postmenopausal women (Kassem et al. 1998) increased serum IGFBP-3 levels. Conversely, immunization of heifers against GH-releasing hormone decreased serum and follicular fluid IGFBP-3 levels (Cohick et al., 1996). Moreover, when Type 1 and 2 diabetic patients were administered insulin and octreotide, a somatostatin analogue, serum levels of IGFBP-3 decreased (Lunetta et al.,1997). As previously mentioned, IGFBP-3 appears as two bands with approximate molecular weights between 40- and 45-kDa. These bands are barely visible in Laron-type (GH-deficient) patients (Gourmelen et al., 1991). Similar stimulatory effects of GH on IGFBP-3 protein and mRNA have likewise been demonstrated in cultured human luteinizing granulosa cells (San Roman and Magoffin, 1993) and rat theca-interstitial cells (Rohan et al., 1993). In contrast, IGFBP-3 mRNA was not influenced by GH treatment in the day 17 bovine uterus, ovary, or oviduct (Kirby et al., 1996). The discrepancy of GH treatment on IGFBP-3 mRNA could be due to the acquirement of mRNA on day 17 after estrus (Kirby et al. 1996), which could compromise GH effects because of GH receptor down-regulation. Moreover, Samaras et al. (1992) found that porcine IGFBP-3 mRNA, primarily found in the CL of the ovary, declined with luteal regression. Hence, the lack of effect of GH on IGFBP-3 expression may also be due to a lack of expression of IGFBP-3 on day 17 of the bovine estrous cycle (Kirby et al. 1996). From this conglomeration of research, GH generally appears to stimulate the production of IGFBP-3.

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Insulin. The effects of insulin on IGFBP-3 production are quite diverse between the cell types. For instance, IGFBP-3 production was enhanced by treatment of cultured porcine granulosa cells with insulin (Grimes and Hammond, 1992) but insulin had no effect on IGFBP-3 protein or mRNA by cultured human retinal pigment epithelial cells (Feldman and Randolph, 1994). Similar responses were observed with IGFBP-3 levels in cultured human fibroblasts (Camacho-Hubner et al., 1992) and granulosa cells (Cataldo et al., 1993). However, serum IGFBP-3 levels were inhibited in streptozocin-induced diabetic pigs (Edwards et al., 1996). These experiments suggest that the affect of insulin on IGFBP-3 levels are influenced by different cell types and species.

Glucagon. Currently, no information is available regarding the effects of glucagon on IGFBP-3 production. Whether glucagon alters ovarian IGFBP-3 is thus unknown.

Hormonal Regulation of IGFBP-4

Growth Factors

IGF. The influence of the IGFs on IGFBP-4 production varies with cell type. IGFBP-4 concentrations increase with IGF-I treatments of cultured rat hepatocytes (Scharf et al., 1996) but are not affected in moderately differentiated cultured porcine granulosa cells (Grimes et al., 1994a). However, in cultured human fibroblasts (for review see, Conover, 1996) and porcine vascular smooth muscle cells (Bourner et al., 1992; Cohick et al., 1993), IGF decreases IGFBP-4 activity presumably by activating a protease to cleave IGFBP-4 (see later section). Irwin et al. (1995) conducted a study in which IGF-I or -II was incubated with IGFBP-4 in a serum-free conditioned medium.

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Immunoblotting indicated that both IGF-I and -II induced a rapid reduction in IGFBP-4 (24-kDa) levels but generated a 16-kDa IGFBP-4 fragment, suggesting direct activation of a protease by the IGFs (Irwin et al., 1995). A similar inhibitory response of IGF-II and IGFBP-4 was observed in cultured human pre-antral follicles (Yuan and Giudice, 1999). Thus, the regulation of IGFBP-4 levels appears to be facilitated by the combination of IGF and proteolytic activity.

EGF. Little work has demonstrated the effects of EGF on IGFBP-4 production, especially in ovarian cells. However, EGF stimulates IGFBP-4 protein and mRNA secretion by cultured human intestinal epithelial cells (Oguchi et al., 1993) and rabbit proximal tubular cells (Yap et al., 1997), respectively. In contrast, no effects by EGF were observed on IGFBP-4 production by cultured human fibroblasts (Conover et al., 1993b). Differences in cell type would likely explain the discrepancies of these results.

bFGF. Little work has evaluated the effects of bFGF on ovarian IGFBP-4. bFGF increased IGFBP-4 production by skeletal cells (Chen et al., 1993) and by cultured human neuroblastoma cells (Babajko et al., 1997) but inhibited production by glia cells (Pons and Torres-Aleman, 1992). Similar to Pons and Torres-Aleman (1992), Hurley et al. (1995) found that expression of IGFBP-4 mRNA and its protein decreased in fetal rat osteoblast cells (Hurley et al., 1995), suggesting the involvement of post-translational mechanisms to regulate IGFBP-4 (Hurley et al., 1995). However, Chen et al. (1993), found that bFGF did not regulate the IGFBP-4 mRNA in primary osteoblast-like cells from fetal rat calvariae. These different outcomes of IGFBP-4 production from bFGF treatments suggest that the discrepancies are due to the different cell models used.

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Gonadotropins

FSH. Cultures of rat granulosa cells with FSH for 24 h stimulated production of IGFBP-4 (Adashi et al., 1991; Adashi et al., 1993). Treatment with FSH, likewise increased IGFBP-4 protein levels in moderately-differentiated porcine (4-6 mm) granulosa cells whereas its message was not (Grimes et al., 1994a). Human pre-antral follicles (Yuan and Giudice, 1999) and rat granulosa cells cultured 72 hours (Liu et al., 1993) in the presence of FSH inhibited IGFBP-4 production. These contrasting results may be explained by a biphasic effect on IGFBP-4 levels (Erickson et al., 1994). Erickson et al. (1994) demonstrated that low doses of FSH (3 ng/ml) increased IGFBP-4 production and high doses (30 and 100 ng/ml) inhibited IGFBP-4 accumulation by cultured rat granulosa cells. Moreover, Western immunoblotting revealed new lower molecular weight bands from the FSH-treated medium (Liu et al., 1993). Unlike FSH-free medium, a 21.5- and 17.5-kDa IGFBP, stained by IGFBP-4 antiserum resulted from FSH treatment (Liu et al., 1993). The 21.5-and 17.5-kDa could have derived from the intact bands of 28- and 24-kDa IGFBP, respectively, which were identified by immunoblotting as IGFBP-4 in rat granulosa cells (Liu et al., 1993). Moreover, 100 ng/ml FSH eliminated IGFBP-4 mRNA in cultured rat granulosa cells (Liu et al., 1993). This suggests that FSH may stimulate granulosa cell maturation by decreasing IGFBP-4 production and allowing the potentiating action of endogenously produced IGF-I.

LH. Addition of 10 ng/ml of hCG to cultured human luteinized granulosa cells increased IGFBP-4 6- to 8-fold when compared to the control (Giudice et al., 1991). Incubation of thecal cells with LH from both large and small ovine follicles also enhanced IGFBP-4 protein and mRNA production (Armstrong et al., 1996; Armstrong et al.,

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1998). The thecal cell study further demonstrated a dose- and time-dependent association of LH treatment on IGFBP-4 enhancement (Armstrong et al., 1996).

Steroids

Estradiol. Estradiol has little effect on IGFBP-4 production. Estradiol had no effect on IGFBP-4 production by cultured human fibroblast cells (Conover et al., 1993b) or porcine granulosa cells (Mondschein et al., 1990). Likewise, exogenous administration of estrogen did not effect the 22-kDa IGFBP (probably IGFBP-4) in plasma of ovariectomized cattle. In contrast, exogenous administration of diethylstilbestrol (DES) to hypophysectomized rats increased IGFBP-4 mRNA levels (Ricciarelli et al., 1991). The variability in these results suggests estradiol effects IGFBP-4 gene expression rather than the protein per se.

Cortisol. Glucocorticoid treatment on IGFBP production results in a number of variable effects by different cell types. For example, dexamethasone and hydrocortisone stimulates IGFBP-4 protein and mRNA secretion by cultured mouse pancreatic β-cells (Katz et al., 1997) and rabbit proximal tubular cells (Yap et al., 1997), respectively. In contrast, both the protein and mRNA levels were decreased with the addition of dexamethasone to cultured human osteoblast cells (Durham et al., 1994b). However, in cultured human fibroblast cells, Conover et al. (1993b) reported no change in IGFBP-4 production. This suggests that IGFBP-4 regulation is dependent upon cell type and treatment utilized.

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Metabolic Hormones

GH. IGFBP-4 levels, in response to GH, are quite varied. GH (0.1 mg/kg) increased follicular fluid and serum IGFBP-4 levels in IVF women patients (Rabinovici et al., 1997) and post-menopausal women (Kassem et al., 1998), respectively, whereas administration of porcine somatotropin (2-4 mg) per day for 6 weeks had no effect on production of the porcine serum or follicular fluid 22-kDa IGFBP (probably IGFBP-4; Echternakamp et al., 1994b). Similarly, treatment with GH antiserum for 8 weeks had no effect on rat IGFBP-4 serum levels (Palmer et al., 1994), and immunization against GH-releasing hormone, which decreases serum GH and IGF-I, has no effect on follicular fluid IGFBP-4 levels in heifers (Cohick et al., 1996). However, co-administration of GH antiserum and bGH doubled rat serum IGFBP-4 levels (Palmer et al., 1994). Unlike IGFBP-5, the increase in IGFBP-4 levels by GH treatment appears to be GH-independent (Thoren et al., 1998). However, in vitro studies have demonstrated that IGFBP-4 production are not influenced by GH in human granulosa cells (Cataldo et al., 1992b), fibroblasts (Conover et al., 1993b) and rat hepatocytes (Scharf et al., 1996), but are stimulated in by GH in rat osteoblast-like cells (Chen et al., 1991).

Insulin. To date, direct effects of insulin on IGFBP-4 production by ovarian cells has not been documented. In cultured human fibroblast cells, Conover et al. (1993b), found no change to IGFBP-4 levels with insulin treatment whereas Camacho-Hubner et al. (1992) demonstrated that insulin enhanced IGFBP-4 production by 3.2-fold. Insulin treatments of 10 ng/ml or less and 100 ng/ml did not affect IGFBP-4 production by human endometrial stromal (Irwin et al., 1995) and neuroblastoma (Babajko et al., 1997) cells, respectively. However, insulin doses of 100 ng/ml and 100 nmol/L applied to

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cultured human endometrial stromal cells (Irwin et al., 1995) and rat hepatocytes (Scharf et al., 1996), respectively, accentuated IGFBP-4 concentrations. Similar studies using cultured porcine vascular smooth muscle cells further support the increases in IGFBP-4 protein and mRNA observed with insulin treatments (Bourner et al., 1992; Cohick et al., 1993).

Glucagon. Few studies have examined the effects of glucagon on IGFBP-4 production. Research that has been performed resulted in no significant effect of glucagon on IGFBP-4 production in cultured rat hepatocytes (Denver and Nicoll, 1994).

Hormonal Regulation of IGFBP-5

Growth Factors

IGF. Addition of IGF-I to rat granulosa cell conditioned medium increased IGFBP-5 production, and the effect was decreased with the addition of FSH (Fielder et al., 1993). The combination of IGF-I (100 ng/ml) and FSH (100 ng/ml) to rat granulosa cells also increased IGFBP-5 production, but with less of an effect than with IGF-I alone. Similar findings with IGF-I were revealed in moderately differentiated porcine granulosa cells from 4-6 mm follicles (Grimes et al., 1994a). Addition of 200 ng/ml of FSH to porcine granulosa cells had no effect on IGFBP-5, but IGF-I-stimulated IGFBP-5 production was decreased in porcine granulosa cells (Grimes et al., 1994a). Therefore, IGF-I prevents the FSH-induced decrease in IGFBP-5 by rat granulosa cells (Fielder et al., 1993).

In cultured human osteoblast cells, IGF-I and -II are the primary modulators of IGFBP-5 (Conover and Kiefer, 1993). The effect of IGF-I was dose dependent as

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demonstrated by a 2.6-fold increase in IGFBP-5 with 0.5 nM IGF-I and 10-fold increase with 10 nM of IGF-I (Conover and Kiefer, 1993). The effect of IGF-I is also time-dependent as demonstrated by the addition of 0.5 mM IGF-I which elicited a 3-fold increase in IGFBP-5 at 2 h and a 10-fold increase at 24 h (Conover and Kiefer, 1993). However, the increase in IGFBP-5 levels by IGF-I did not reflect changes in IGFBP-5 mRNA expression (Conover and Kiefer, 1993). Similar findings of increased activity of IGFBP-5, but not its message, due to the addition of and exposure to IGF-I were revealed in cultured human fibroblast cells (Camacho-Hubner et al., 1992). Thus, IGFBP-5 appears to be posttranscriptionally regulated by IGF-I in cultured human osteoblast (Conover and Kiefer, 1993) and fibroblast (Camacho-Hubner et al., 1992) cells. To increase IGFBP-5 in vitro, the protein must be associated with IGF-I and/or -II, not the IGF receptor (Camacho-Hubner et al., 1992). It is postulated that IGF-I binds to secreted IGFBP-5, partially protecting it against hydrolysis by an IGFBP-5 protease (Conover and Kiefer, 1993; Nam et al., 1994). Thus, in the absence of IGF-I or -II, IGFBP-5 undergoes proteolysis (for review see, Conover, 1996).

EGF. Few studies have involved EGF regulation of the IGFBPs. However, one study has demonstrated that IGFBP-5 mRNA production was decreased in the presence of EGF in cultured rabbit proximal tubular cells (Yap et al., 1997).

bFGF. Like EGF, little ovarian work has detailed the influence of bFGF with IGFBP-5. However, both IGFBP-5 and bFGF have prominent roles in bone cell cultures. bFGF functions to stimulate bone cell replication (Canalis et al., 1993; Canalis and Gabbitas, 1995) while IGFBP-5 serves to enhance bone cell growth and stimulate the anabolic actions of IGF-I (Andress and Birnbaum, 1992). Treatment of cultured fetal

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rat osteoblast cells with bFGF for 6 to 24 h diminished IGFBP-5 protein, as well as its gene expression, and IGF-I activity (Canalis et al., 1993; Canalis and Gabbitas, 1995).

Gonadotropins

FSH. Production of IGFBP-5 by ovine cultured granulosa cells was not affected by FSH treatment (Monget et al., 1998). However, in cultured rat granulosa cells, low doses of ovine FSH (1-3 ng/ml) increased a 28-29 kDa IGFBP, representing IGFBP-5, whereas high doses of FSH (>10 ng/ml and 100 ng/ml; Adashi et al., 1990a; Adashi et al., 1991; Fielder et al., 1993) inhibited IGFBP-5. Another lower molecular weight IGFBP (21.0-kDa) was detected by Western immunoblots from granulosa cells with FSH-treated medium which could be derived from the intact 29-kDa band of IGFBP-5 (Liu et al., 1993). Northern blot analysis also revealed that 100 ng/ml of FSH decreased IGFBP-5 mRNA in cultured rat granulosa cells (Liu et al., 1993). This suggests that FSH works at the gene level to inhibit IGFBP-5 transcription or potentiate degradation of IGFBP-5 mRNA (Liu et al., 1993).

LH., Thecal and granulosa cell LH/hCG receptor numbers increase in the early dominant follicle during the bovine estrous cycle (Xu et al., 1995b; Bodensteiner et al., 1996). Because bovine follicular fluid IGFBP-5 levels are negatively correlated with the number of thecal LH/hCG binding sites, a regulatory role of LH by IGFBP-5, as well as IGFBP-2, and -4, is possible (Stewart et al., 1996). However, another study found that LH increased IGFBP-5 mRNA production by cultured human thecal cells (Voutilainen et al., 1996). Although these results are conflicting, the data supports the concept that gonadotropins may regulate IGFBP-5 production in thecal tissue.

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Steroids

Estradiol. Although few studies have determined the effects of estradiol on IGFBP-5 levels follicular fluid, a relationship between these two factors seems to exist. Stewart et al. (1996) demonstrated that estradiol concentrations were negatively correlated with IGFBP-5 concentrations in dominant and large bovine follicles. Similar results were found in estrogen-active preovulatory follicles of women (Schuller et al, 1993). However, treatment of human cultured osteoblast cells with estradiol (100 nM) had no effect on IGFBP-5 production (Conover and Kiefer, 1993). This discrepancy is likely due to the cell-type studied within each experiment.

Cortisol. Glucocorticoids attenuate DNA and collagen synthesis in bone cultures and decrease IGF-I synthesis in osteoblasts (McCarthy et al., 1990; Delany et al., 1994; Okazaki et al., 1994), so an interaction between IGFBP-5, a predominant IGFBP in bone, and cortisol seems logical. Treatment of cortisol decreased IGFBP-5 production in the extracellular matrix of cultured fetal rat osteoblast cells (Gabbitas et al., 1996). IGFBP-5 mRNA levels were also decreased in a time- and dose-dependent manner by cortisol treatments which would suggest IGFBP-5 in fetal rat cultured osteoblast cells is regulated transcriptionally by cortisol (Gabbitas et al., 1996). Similar studies using cultured human fibroblasts (Camacho-Hubner et al., 1992) and osteoblast cells (Conover and Kiefer, 1993) showed that dexamethasone treatments decreased or had no effect on IGFBP-5 levels, respectively. Moreover, hydrocortisone increased IGFBP-5 mRNA by rabbit proximal tubular cells (Yap et al., 1997). Therefore, species and/or cell-type likely influences whether IGFBP-5 production is altered by glucocorticoids.

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Metabolic Hormones

GH. Immunization of cattle against GH-releasing hormone, which decreases serum GH and IGF-I, has no effect on follicular fluid IGFBP-5 levels (Cohick et al., 1996). A study conducted by Thoren et al. (1998) illustrated that serum IGFBP-5 levels were significantly diminished in GH-deficient patients when compared to control patients. Upon 36 months of GH-therapy, serum IGFBP-5 levels increased by 2-fold after the third month of treatment with the GH-deficient subjects (Thoren et al., 1998). However, IGF-I levels were also elevated which suggest that the increase in IGFBP-5 is not due to GH but IGF-I (Thoren et al., 1998). In support of this suggestion, no change was detected in IGFBP-5 protein levels with GH treatment (1 μg/ml) to human osteoblast cells in vitro (Conover and Kiefer, 1993). However, GH increased IGFBP-5 mRNA levels in cultured fetal rat osteoblast cells (McCarthy et al., 1994).

Insulin. The effect of insulin on IGFBP-5 production was determined because insulin can bind to the IGF-I receptor, and IGF-I and –II significantly enhance IGFBP-5 activity in human fibroblasts (Camacho-Hubner et al., 1992). However, treatment with insulin to cultured human fibroblasts (Camacho-Hubner et al., 1992) and osteoblast cells (Conover and Kiefer, 1993) resulted in no significant effect on IGFBP-5 levels. These results indicate that IGF-I and –II must associate with IGFBP-5 to increase IGFBP-5, and that receptor binding is not required (Camacho-Hubner et al., 1992).

Glucagon. Currently, no information is available regarding the effect of glucagon on IGFBP-5 production.

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Hormonal Regulation of IGFBP-6

Growth Factors

IGF. IGFBP-6, which binds IGF-II with 20-100 times greater affinity than IGF-I, selectively prevents the effects of IGF-II on myoblasts and osteoblasts (Kiefer et al., 1992; Bach et al., 1993; 1994). However, IGF-II had no effect on IGFBP-6 gene expression by fetal rat osteoblast cells (Gabbitas and Canalis, 1997). Likewise, IGF-I had no effect on IGFBP-6 mRNA expression in cultured fetal rat osteoblast cells (Gabbitas and Canalis, 1997) or human retinal pigment epithelial cells (Feldman and Randolph, 1994). The lack of effect of IGF on mRNA would suggest IGFBP-6 is not regulated by transcriptional mechanisms. However, the modulation of IGF-II by IGFBP-6 suggests that changes in IGFBP-6 have the capability to alter the available levels of IGF-II in skeletal muscle and other tissues where both are produced (i.e., ovary).

EGF. Similar to the lack of effect of IGF-II on IGFBP-6 production, EGF had no effect on IGFBP-6 mRNA expression, but slightly increased IGFBP-6 protein levels in cultured human retinal pigment epithelial cells (Feldman and Randolph, 1994). Thus, within this cell-line EGF may have a minor role in the regulation of IGFBP-6. Whether EGF plays a role in the regulation of ovarian IGFBP-6 is unknown.

bFGF. Unlike IGFBP-1 to 5, bFGF had little effect on IGFBP-6 activity. For example, IGFBP-6 mRNA and protein concentrations were not affected by treatment of bFGF in reports using fetal rat osteoblast (Gabbitas and Canalis, 1997) or cultured human neuroblastoma (Babajko et al., 1997) cells. Thus, from these few studies, bFGF doesn't appear to regulate IGFBP-6 production. Whether bFGF alters ovarian IGFBP-6 production remains to be determined.

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Gonadotropins

FSH. Generally, granulosa cell-derived IGFBPs are reduced by FSH (Adashi et al., 1990a). When hypophysectomized rats were treated with FSH, whole ovarian IGFBP-6 transcripts decreased by 2.4-fold relative to that in hypophysectomized controls (Rohan et al., 1993). However, unlike most mammalian species, the antigonadotropic potential of rat IGFBP-6 mRNA primarily works at the level of the thecal cells rather than the granulosa cells (Rohan et al., 1993). Increasing concentrations of rhIGFBP-6 did not inhibit the FSH-supported accumulation of either progesterone or estradiol by rat granulosa cells (Rohan et al., 1993). This suggests that the failure of rhIGFBP-6 to diminish FSH action by granulosa cells is due to its comparatively reduced affinity for IGF-I (Kiefer et al., 1992).

Steroids

Estradiol. Rat IGFBP-6 gene expression is primarily within the theca-interstitial cells, with little or no expression in granulosa cells (Rohan et al., 1993). Evidence suggests estradiol inhibits IGFBP-6 production by ovarian cells. Administration of DES to hypophysectomized rats resulted in a 2.6-fold decrease in IGFBP-6 transcripts (Rohan et al., 1993). Because IGFBP-6 mRNA in rat theca-interstitial cells was inhibited by estrogen (Ricciarelli et al., 1992; Rohan et al., 1993), estrogen's effect may be through a direct influence on theca-interstitial cells (Rohan et al., 1993). However, estrogen may also effect IGFBP-6 transcript levels through a granulosa cell-derived intermediate (Rohan et al., 1993). Because FSH and estrogen enhance many rat granulosa cell functions (Richards and Hedin, 1988), the latter idea is further supported by results

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which show the combination of FSH and DES synergistically decrease the amounts of whole ovarian IGFBP-6 transcripts (Rohan et al., 1993).

Cortisol. Currently, no information is available regarding the effects of cortisol on the production of IGFBP-6.

Metabolic Hormones

GH. Treatment of hypophysectomized rats with GH had no effect on IGFBP-6 mRNA levels in theca-interstitial cells (Rohan et al., 1993) or on systemic IGFBP-6 levels (Lee et al., 1997). GH treatment of fetal rat osteoblast cells in vitro also had no effect on IGFBP-6 mRNA (McCarthy et al., 1994).

Insulin. Unlike the other IGFBPs, IGFBP-6 is not impacted by insulin treatments. As exemplified in cultured human retinal pigment epithelial (Feldman and Randolph, 1994) and neuroblastoma cells (Babajko et al., 1997), insulin had no effect on IGFBP-6 mRNA or protein levels, respectively. Thus, as indicated at least from these cell culture types, insulin does not regulate the production of IGFBP-6. The effect on ovarian cells has not been determined.

Glucagon. Currently, no information regarding the effect of glucagon on IGFBP-6 production is available.

Proteolysis of the IGFBPs

Posttranslational modifications decrease binding protein affinity for their peptide ligands. As previously alluded to, proteolytic activity is one such mechanism that may modulate the levels of IGFBPs within various tissues. Proteolysis of the IGFBPs was originally described in the circulation of pregnant women (Giudice et al., 1990a;

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Hossenlopp et al., 1990). However, subsequent work has shown that modified or cleaved binding proteins are present in most, if not all extracirculatory fluids (Lalou and Binoux 1993; Dodd et al., 1995; Xu et al., 1995a; Matsumoto et al., 1996). A prime example of IGFBP proteolysis can be found in the sheep ovary. In ovine follicular fluid, proteases are produced for IGFBP-4, and to an extent for IGFBP-2, -3, and -5 (Monget et al., 1996). During terminal follicular growth of antral follicles, proteolytic activity of IGFBP-3 is reduced while proteolysis of IGFBP-2, -3, and -5 is enhanced (Monget et al., 1996). During atresia, IGFBP-3 proteolytic activity in small (2-3 mm) ovine follicles increases whereas IGFBP-4 and -5 proteolytic activity in large (>5 mm) follicles decrease (Monget et al., 1996).

Proteolysis of the IGFBPs is believed to modulate IGF bioavailability. Theoretically, by cleaving the intact IGFBPs into fragments, the resulting degradation could release bound IGF from the binding proteins, thus leading to follicular growth and maturation (Grimes and Hammond, 1994). The majority of these proteolytic fragments of the various IGFBPs do not bind IGF-I (Rajaram et al., 1997). Conversely, a decrease in IGFBP degradation could stabilize extracellular IGFs and fine tune IGF activity as suggested by Grimes and Hammond (1994).

As mentioned earlier, IGF-I may stimulate IGFBP proteolysis. Iwashita and colleagues (1996) suggested three possible mechanisms by which IGFs induce proteolysis. These include either direct activation of a latent protease by IGFs, increased susceptibility of IGF/IGFBP complexes to proteolytic degradation and/or repression of a protease inhibitor by the IGFs. Which, if any, theory is correct, is yet to be determined. However one postulated theory about increasing the availability of the IGFs involves the

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interaction of IGFBP-3 and the low molecular weight binding proteins (for review see, Rajaram et al., 1997). Most IGFs are complexed to IGFBP-3 in the serum. An IGFBP-3 protease present in serum or vascular endothelial cells can cleave IGFBP-3 and therefore release IGF. As previously mentioned, the binding of IGF-I to IGFBP-3 increases the half-life of IGF-I from 20-30 minutes in the free form to approximately 15-20 hours in the bound form (Zapf et al., 1986). Once the IGFs dissociate from IGFBP-3, they either remain free, or most likely, bind to a lower molecular weight IGFBP and cross the endothelium. Once IGF is transported into the tissue space, a protease may degrade the low molecular weight IGFBP, thus releasing and enabling IGF-I and -II to bind to their receptors. Therefore, the IGFBP protease may modulate the transport of IGFs into tissue space, regulate the availability of free IGFs and alter the metabolic clearance rate of IGFs. However, proteolysis can not fully explain how IGF passes the endothelial barrier since intact IGFBP-3 has been found in the follicular compartments. An alternative hypothesis suggests that during preovulatory maturation, intrafollicular IGFBP-3 activity is increased because of increased vascularization and permiability of the follicle during follicular growth, the entrance of serum-derived IGFs and IGFBPs may ensue (Grimes et al., 1994).

IGFBP-2 Proteolysis

Although little information regarding IGFBP-2 proteolysis is available, indirect evidence of IGFBP-2 protease activity has been demonstrated in bovine (Stanko et al., 1994), ovine (Besnard et al., 1996b) and porcine (Besnard et al., 1997) follicular fluid, human thecal- and stromal-conditioned medium (Mason et al., 1996) and porcine smooth muscle cells (Cohick et al., 1995). Stanko et al. (1994) reported that in bovine follicular

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fluid of dominant and subordinate follicles, two non-IGF binding IGFBP-2 fragments, approximately 22- and 14-kDa, existed along with the intact IGFBP-2 (34-kDa). Similarly, Mason et al. (1996) reported and intact IGFBP-2 band at 35-kDa as well as several lower molecular mass bands at 33-, 32-, 25-, 23-, 20.5-, and 16-kDa, detected by immunoblotting, in human thecal- and stromal-conditioned medium. These bands were postulated to be proteolytically cleaved products (Mason et al., 1996).

Besnard et al. (1996b) found that ovine follicular fluid from different classes of follicles, likewise contained specific proteolytic activity degrading IGFBP-2. Indeed, ovine follicular growth (between 2-6 mm) was characterized by an increase in IGFBP-2 proteolytic activity, as detected by immunoblotting (Besnard et al., 1996b). Specifically, IGFBP-2 proteolysis was slightly greater in medium sized follicles than in small follicles (Besnard et al., 1996b). However, proteolytic activity in ovine medium follicles was similar in atretic and healthy follicles (Besnard et al., 1996b). Although IGFBP-2 proteolysis was detected in ovine follicles, IGFBP-3 and -4 are the predominant IGFBPs undergoing proteolysis (both IGFBP-2 and -5 are degraded to a lesser extent) in the sheep (Besnard et al., 1996b). Thus, few studies have attempted to specifically identify the IGFBP-2 protease in ovine follicles.

Immunoblot analyses indicated an increase in a 22- and 14-kDa non-IGF binding IGFBP-2 fragment as well as a decrease of intact IGFBP-2 in porcine smooth muscle cells subsequent to IGF-II treatment (Cohick et al., 1995). Treatment with IGF-I also increased the appearance of the IGFBP-2 fragments, but a corresponding decrease of intact IGFBP-2 did not result (Cohick et al., 1995). In contrast, insulin treatment had no effect on IGFBP-2 fragmentation by porcine smooth muscle cells (Cohick et al., 1995).

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In addition, porcine follicular fluid from growing follicles 2-7 mm in diameter contained proteolytic activity for IGFBP-2 whereas atretic follicles were associated with a marked decrease in IGFBP-2 proteolytic activity (Besnard et al., 1997). Indeed, incubation of IGFBP-2 with porcine preovulatory follicular fluid resulted in the appearance of a 16-kDa fragment as detected by immunoblotting (Besnard et al., 1997). However, inhibition of IGFBP-2 proteolysis resulted with the addition of EDTA (calcium chelator) or 1,10 phenanthroline (chelator of zinc ions) in porcine follicular fluid from preovulatory follicles (Besnard et al., 1997). The addition of calcium chloride and zinc chloride was necessary to completely restore IGFBP-2 proteolytic activity inhibited by EDTA (Besnard et al., 1997). PMSF (serine protease inhibitor), aprotinin (serine protease inhibitor), benzamide (serine protease inhibitor), E64 (cysteine protease inhibitor), phoshoramidon (endopeptidase inhibitor), and leupeptin (trypsin-like serine and cysteine protease inhibitor) resulted in little to no inhibition of porcine IGFBP-2 proteolytic inhibition (Besnard et al., 1997). These results indicate that the protease(s) involved in the degradation of IGFBP-2 in porcine follicular fluid belong to the metalloprotease family and are zinc- and calcium-dependent (Besnard et al., 1997).

IGFBP-3 Proteolysis

IGFBP-3 protease activity has been found in a variety of biological fluids, including ovine follicular fluid and human (Giudice et al., 1990b; Hossenlopp et al., 1990; Davies et al., 1991), pig (Besnard et al., 1997), sheep (Besnard et al., 1996b), rat (Davenport et al., 1990; Gargosky et al., 1990), and mouse (Fielder et at., 1990) pregnancy serum, as well as the human prostate (Cohen et al.,1992) and porcine granulosa cells (Grimes and Hammond, 1994). Furthermore, after the second month of

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pregnancy in humans, a significant decrease in binding protein activity, particular IGFBP-3, has been observed. This decrease may be due to a serum protease which degrades IGFBP-3 into fragments, releasing sequestered IGF for biological activity. When IGFBP-3 undergoes proteolysis, low molecular weight fragments, detected by Western immunoblots, appear; these fragments do not bind IGF-I or -II. In cultured human thecal explants from dominant follicles, intact IGFBP-3 (41.5- and 44-kDa) as well as a lower molecular weight doublet (30- and 32-kDa) were detected by immunoblotting (Cwyfan-Hughes et al., 1997). The proportion of IGFBP-3 appearing as a 30- and 32-kDa doublet increased from 2 to 78% as the size of the dominant follicle increased (Cwyfan-Hughes et al., 1997).

Protease activity degrading IGFBP-3 was demonstrated in ovine follicular fluid, primarily from small atretic follicles (Besnard et al., 1996b). Degradation of ovine follicular fluid IGFBP-3 was blocked by EDTA and 1,10 phenanthroline. However, EDTA's effects was partially reversed by the addition of calcium chloride and completely opposed by the addition of both calcium and zinc chloride whereas the effects of 1,10 phenanthroline was negated with the addition of zinc chloride (Besnard et al., 1996b). These results suggest that intrafollicular ovine IGFBP-3 proteolytic activity, similar to that of IGFBP-2 in pigs, involves calcium- and zinc-dependent metalloproteases (Besnard et al., 1996b).

Porcine follicular fluid contains small amounts of proteolytic activity specific for IGFBP-3 (Besnard et al., 1997). Grimes and Hammond (1994) demonstrated that the protease appears to be a soluble serine-specific protease, similar to plasmin, produced by porcine granulosa cells. When cultured porcine granulosa cells were treated with 20

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μg/ml aprotinin, a serine protease inhibitor, 100% rhIGFBP-3 (29-kDa) remained intact (Grimes and Hammond, 1994). In contrast, the addition of the cysteine-specific inhibitor trans-epoxy succinly-L-leucyl-amido-(4-guanidino) butane, aspartic-specific inhibitor pepstatin, or the metalloprotein-specific inhibitor 1, 10-phenanthroline, did not maintain intact IGFBP-3 (Grimes and Hammond, 1994). Furthermore, plasmin and plasminogen, an inactive precursor to plasmin, degrades rhIGFBP-3 into non-IGF-I binding fragments of approximately 14.3-, 18.5-, 22.5-, and 24-kDa. (Grimes and Hammond, 1994). Similarly, plasmin has been shown to degrade IGFBP-1 in osteoblast cultures (Campbell et al., 1992).

Regulation of IGFBP-3 proteolysis is still in question, but is believed to be hormonally controlled. Bang and colleagues (1994) demonstrated that insulin may be involved in regulating IGFBP-3 proteolysis. In non-insulin dependent diabetes mellitus patients that were administered insulin, IGFBP-3 protease activity decreased (Bang et al., 1994). Furthermore, Vinter-Jensen et al. (1996) hypothesized that because EGF stimulates IGF-I biosynthesis in the liver (Barreca et al., 1992), systemic EGF affects the post-transcriptional processing of the IGF/IGFBP-3 complex by inducing proteolytic activity. However, Western immunoblots revealed that insulin (40% intact rhIGFBP-3) or EGF (26% intact rhIGFBP-3) treatments had no significant effect on rhIGFBP-3 proteolysis, when compared to the controls (37% intact rhIGFBP-3) in cultured porcine granulosa cells (Grimes and Hammond, 1994). Likewise, FSH (29%), estradiol (20%), and GH (23%) did not significantly effect proteolysis (Grimes and Hammond, 1994). Unlike the aforementioned hormones used by Grimes and Hammond (1994), the addition of IGF-I or -II to the porcine granulosa cell cultures, diminished proteolysis of rhIGFBP-

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3 (Grimes and Hammond, 1994). Addition of genistein, a tyrosine kinase inhibitor that blocks the action of the type I receptor, can counteract IGF-I, as well as insulin stimulation of endogenous porcine IGFBP-3 production (Grimes and Hammond, 1994). However, administration of genestein to the porcine granulosa cell cultures did not block the IGF-I-induced inhibition of the degradation of rhIGFBP-3 (Grimes and Hammond, 1994). Moreover, because insulin was ineffective in preventing degradation of rhIGFBP-3, the insulin receptor is also an unlikely candidate to modulate protease activity (Grimes et al., 1994). The type II receptor is also improbable because IGF action within the porcine ovary appears to be through the type I receptors (Adashi et al., 1990b; Nissley and Lopaczynski, 1991). Because of these factors, a non-receptor-mediated action to regulate IGFBP proteases of the IGFs must be considered. One possibility of IGF action is the stabilization of IGFBP-3 from proteolysis by binding to IGFBP-3.

IGFBP-4 Proteolysis

IGFBP-4 protease activity has been reported in porcine (Cohick et al., 1993) and human (Kamyar et al., 1994) smooth muscle cells, human osteoblast-like cells (Durham et al., 1994a), fibroblasts (Fowlkes and Freemark, 1992; Conover et al., 1993b), endometrial stromal cells (Irwin et al., 1995), decidua (Myers et al., 1993), and dominant estrogenic follicles (Chandrasekher et al., 1995), rat neuronal cell lines (Cheung et al., 1994) and granulosa cells (Erickson et al., 1994), porcine (Besnard et al., 1997) and ovine follicular fluid (Besnard et al., 1996b; Mazerbourg et al., 1999), and both human and ovine fibroblasts (Conover et al., 1993b; Neely and Rosenfeld, 1992; Fowlkes and Freemark, 1992). Binding of the IGFs to IGFBP-4 increases the susceptibility of this

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binding protein to proteolysis, possibly as the result of a conformational change (Mohan et al., 1994).

Similar to porcine IGFBP-2, a protease degrading IGFBP-4 from porcine follicular fluid exists in growing follicles 2 mm and 6-7 mm in diameter (Besnard et al., 1997). However, the addition of EDTA and 1,10 phenanthroline to porcine follicular fluid samples inhibits degradation of IGFBP-4 (Besnard et al., 1997). Likewise, in porcine smooth muscle cells, an intact 24-kDa IGFBP, coinciding with nonglycosylated IGFBP-4 is proteolytically cleaved to a 16-kDa fragment that is not capable of binding IGF-I/-II (Cohick et al., 1993). Exposure of these cells to IGF-I further increases the amount of IGFBP-4 fragments whereas exposure to insulin has no effect on IGFBP-4 proteolysis (Cohick et al., 1993).

Degradation of IGFBP-4 in ovine follicular fluid is the primary IGFBP undergoing proteolysis in preovulatory follicles (Besnard et al., 1996b). Degradation of IGFBP-4 was completely blocked by chelating agents (EDTA and 1,10 phenanthroline; Besnard et al., 1996b). Moreover, the addition of calcium chloride and/or zinc chloride overrode the inhibition by the chelating agents, resulting in degradation of ovine IGFBP-4 (Besnard et al., 1996b). Thus, similar to the follicular fluid protease found to degrade IGFBP-2 and -3 in sheep and pigs, the IGFBP-4 protease is a calcium- and zinc-dependent metalloprotease.

In human fibroblasts and human osteoblast-like cells, the cleavage site of IGFBP-4 is located in domain 2, which is the central nonconserved region of the binding proteins (Conover et al., 1995). The conserved domains 1 and 3, which are the NH_2 and COOH-terminal portions of the binding proteins are important for IGF binding (Conover et al.,

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1995). Therefore, the nonconserved domain 2 could be involved in regulating the activity and /or tissue specificity of each IGFBP (Conover et al., 1995).

Conover et al. (1993b), demonstrated that human fibroblasts secrete and IGFBP-4 specific protease that is dependent upon IGFs for activation. Research has shown that agents that complex divalent cations block the IGF-induced decrease in IGFBP-4 (Conover et al., 1993b). Furthermore, the protease involved in degrading IGFBP-4 is specific for that protein. Conover et al. (1993b) reported that cell-free incubation with or without IGF-II had no effect on other binding proteins present in human fibroblast conditioned medium or on IGFBP-1, -2, -3, -5, or -6, when added to the conditioned medium. Therefore, the IGFBP-4 protease is unlikely to be the same as the cation-dependent IGFBP-3 protease. Although the identity of the protease for IGFBP-4 remains unknown, IGFBP-4 protease levels are reduced in the presence of the metalloserine protease inhibitor, EDTA (a divalent cation chelator), the serine protease inhibitor aprotinin, and the metal ion chelator 1,10 phenanthroline, in human endometrial stromal cells (Irwin et al., 1995) and human follicular fluid (Chandrasekher et al., 1995). Other proteinase inhibitors including leupeptin, E64, $\alpha_1$trypsin inhibitor, pepstatin and chymostatin were ineffective at inhibiting protease activity in human endometrial stromal cells (Irwin et al., 1995).

Conover et al. (1995) suggested a role for kallikrein in the degradation of IGFBP-4. These researchers demonstrated that the IGFBP-4 protease cleaves the intact IGFBP-4 molecule at a single site on the carboxyl terminal side of methionine 135, producing two fragments (18- and 14-kDa). The 14-kDa product is a result of cleavage between methionine and lysine IGFBP-4. Consequently, tissue kallikreins are known to

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cleave peptide bonds between methionine and lysine and also between arginine and serine in kininogen to release lyslybradykinin. Therefore, IGFBP-4 cleavage may be a result of a hydrolytic enzyme with a specificity similar to that of kallikrein.

Chandresekher et al. (1995) demonstrated that IGFBP-4 protease activity in human follicular fluid was pH and temperature dependent. A temperature range of 25-37°C, but not 4°C, will induce protease activity, with maximal IGFBP-4 reduction occurring at 37°C (Chandrasekher et al., 1995). Likewise, optimal protease activity ensues at a pH range of 7-9, but not 4-5 (Chandrasekher et al., 1995).

An IGFBP-4 protease is present in human dominant estrogenic, or healthy follicles, but not androgen-dominant, or atretic follicles (Chandrasekher et al., 1995). However, FSH and estradiol has also been shown to induce IGFBP-4 proteolysis in human granulosa cell cultures (Iwashita et al., 1996). Whether estradiol and FSH directly stimulate the production of a protease by granulosa cells or activate proteolytic activity through IGF-mediated mechanisms, such as repression of a proteolytic inhibitor by IGF-I, remain unknown. However, the addition of IGF-I, but not FSH, to granulosa cell cultures stimulated proteolysis of ^{125}I-IGFBP-4 (Iwashita et al. 1998). This suggests that increased susceptibility of IGFBP-4 to proteolysis is induced by the binding of IGFs to IGFBP-4 (Iwashita et al., 1998).

The regulation of IGFBP-4 degradation is also influenced by the IGFBPs in human follicular fluid. By adding unsaturated IGFBP-1, -2, -3, or more effectively, IGFBP-4, IGFBP-4 protease activity is significantly reduced, as depicted through Western immunoblots and scanning densitometry techniques (Cwyfan-Hughes et al., 1997). Furthermore, by preincubating IGFBP-3 with an equimolar amount of IGF-I, the

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inhibition of IGFBP-4 protease activity can be reversed (Fowlkes et al., 1995; Cwyfan-Hughes et al., 1997). Therefore, in atretic follicles, where IGF-I concentrations are low in human follicular fluid, the majority of IGFBP-3 is unsaturated and can therefore block the IGFBP-4 protease, thus resulting in IGFBP-4 inhibiting IGF activity. Conversely, in healthy dominant follicles, the increased concentration of IGF would result in most of IGFBP-3 being saturated with IGF and thus unable to counteract IGFBP-4 protease activity. The fragmented IGFBP-4, not detectable by Western ligand blotting, has a decreased affinity for IGF-I and thus can not inhibit IGF activity (Conover et al., 1993b). IGF-I becomes more available to the cell receptors which enables the follicle to proceed into final maturation (Cwyfan-Hughes et al., 1997). Using murine osteoblasts, Fowlkes et al. (1995) further elucidated that the inhibitory domain in IGFBP-3 may be located in the non-homologous mid-region of the molecule and/or in the C-terminal domain (Fowlkes et al., 1995). However, in murine osteoblasts, IGFBP-4 can be degraded without the addition of IGF-I (Fowlkes et al., 1995).

Durham et al. (1994b) further demonstrated the effects of growth factors in the degradation of IGFBP-4 in normal human osteoblast-like cells. Incubation of ^{125}I-rhIGFBP-4 with the addition of IGF-II decreased the amount of intact IGFBP-4 and increased the amount of two fragments with molecular weights of 18- and 14-kDa (Durham et al., 1994b). IGF-I likewise decreased IGFBP-4 levels but was not as effective as IGF-II (Conover et al., 1993b; Durham et al., 1994). Durham et al. (1994b) also demonstrated that in the presence of IGF-II, TGFβ further increased IGFBP-4 proteolysis. TGFβ appears to indirectly enhance IGF-dependent IGFBP-4 proteolysis by stimulating osteoblast-like cell expression and/or secretion of a latent IGFBP-4 protease

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(Durham et al., 1994b). IGF-II may initiate IGFBP-4 protease activity by directly binding to IGFBP-4 or inactivating an inhibitor to IGFBP-4 proteolysis (Durham et al., 1994b). Therefore, IGFBP-4 activity in human osteoblast-like cells can be regulated by IGF-II and TGFβ (Durham et al., 1994b), the two growth factors that are abundant in bone. In contrast, Durham et al. (1994b) found that GH, insulin, or estradiol, did not alter IGFBP-4 protease activity in human osteoblast-like cells. Likewise, treatment with EGF also did not alter IGF-dependent IGFBP-4 proteolysis. In rat neuronal cells, glucocorticoids have also been reported to induce IGFBP-4 protease activity (Cheung et al., 1994). Whether these hormones alter ovarian cell IGFBP-4 protease activity remains to be elucidated.

IGFBP-5 Proteolysis

IGFBP-5 protease activity has been demonstrated in human osteosarcoma cells (Conover and Kiefer, 1993) and fibroblasts (Camacho-Hubner et al., 1992), serum of neonatal fasted pigs (McCusker et al., 1991b), porcine smooth muscle cells (Cohick et al., 1993), bovine (Stanko et al., 1994), ovine (Besnard et al., 1996b; Monget et al., 1996) and porcine (Besnard et al., 1997) follicular fluid and rat granulosa cells (Fielder et al., 1993). Calcium-dependent serine proteases and matrix metalloproteases degrade IGFBP-5 in bone cells (Gabbitas et al., 1996). In addition, the proteolytic enzyme to IGFBP-5 produced by rat granulosa cells is cation-dependent (Fielder et al., 1993).

Evidence using Western immunoblotting techniques, suggests a protease for IGFBP-5 is apparent in human osteosarcoma cells. During incubation in cell-conditioned medium, the intact 29-kDa (nonreduced) and 34-kDa (reduced) intact bands for rhIGFBP-5 are lost and a 16-, 17- and 20-kDa band appears (Conover and Kiefer,

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1993b). The addition of either IGF-I or -II increased the intact IGFBP-5, however, the amount of the fragments were not decreased. Therefore, the addition of IGF-I or -II does not protect IGFBP-5 from proteolysis.

In bovine follicular fluid, a 21-kDa fragment, reacting to the IGFBP-5 antiserum was detected using Western immunoblotting, along with the intact IGFBP-5 doublet (31-32-kDa; Stanko et al., 1994). These two fragments related in size with fragments that have been identified in serum of neonatal fasted pigs (McCusker et al., 1991b) as well as in media conditioned by pig smooth muscle cells (Cohick et al., 1993) and human fibroblasts (Camacho-Hubner et al., 1992).

Ovine and porcine follicular fluid likewise contains a protease that degrades IGFBP-5 (Besnard et al., 1996b). During follicular growth IGFBP-5 proteolytic activity increases slightly and decreases in large atretic follicles (Besnard et al., 1996b; Besnard et al., 1997). The progressive increase in proteolytic activity degrading IGFBP-5 is in agreement with previous results obtained by Western ligand blotting showing that ovine and porcine follicular growth is associated with a decrease in IGFBP-5 (Besnard et al., 1996a; Besnard et al., 1997). Moreover, degradation of IGFBP-5 by ovine follicular fluid was completely inhibited by EDTA and 1,10 phenanthroline and partially restored with the addition of calcium chloride and/or zinc chloride (Besnard et al., 1996b; Besnard et al., 1997). Thus, based on work by Besnard et al. (1996; 1997), sheep and pig follicular fluid contains one or more metalloproteases that degrade IGFBP-2, -3, -4, and -5.

Various hormones and growth factors may also modulate protease activity for IGFBP-5. For example, in bone cells, cortisol may enhance IGFBP-5 protease activity

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(Gabbitas et al., 1996). Likewise, in rat granulosa cells, FSH (Fielder et al., 1993; Liu et al., 1993) induces production of an IGFBP-5 protease. However, IGF-I or -II (Fielder et al., 1993) blocks this proteolytic activity, as also demonstrated in fibroblast-conditioned medium (Camacho-Hubner et al., 1993). Therefore, FSH may induce an IGFBP-5 protease, or alternatively, FSH may decrease an IGFBP-5 protease inhibitor, leading to the activation of the IGFBP-5 protease.

IGFBP-6 Proteolysis

Unlike the other binding proteins, fewer investigations of proteases for the more recently identified IGFBP-6 have been reported. However, like the aforementioned binding proteins, IGFBP-6 is also affected by proteolytic activity. Neumann et al. (1998), who demonstrated O-glycosylation of IGFBP-6, also investigated degradation of nonglycosylated and glycosylated IGFBP-6. When chymotrypsin was incubated with IGFBP-6, 1 ng and 10 ng chymotrypsin cleaved intact nonglycosylated and glycosylated IGFBP-6, respectively (Neumann et al., 1998). Clearly, additional research is needed to determine if IGFBP-6 proteolytic enzymes exist within the ovarian follicle.

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Table 1

Binding affinities of the various IGFBPs for IGF-I and IGF-II

	MW (kDa)	Relative Affinity	Affinity Constants for IGF-I[a]	Affinity Constants for IGF-II[a]
IGFBP-1	25-34	IGF-II > IGF-I	6×10^{9}	1.2×10^{10}
IGFBP-2	29-40	IGF-II > IGF-I	1.3×10^{9}	2.3×10^{9}
IGFBP-3	150[b]	IGF-I = IGF-II	1×10^{10}	1.2×10^{10}
IGFBP-4	24-30	IGF-I = IGF-II	2×10^{10}	1.9×10^{10}
IGFBP-5	29-31	IGF-I = IGF-II	2.6×10^{10}	2.3×10^{10}
IGFBP-6	21-32	IGF-II >> IGF-I	8×10^{8}	1.7×10^{10}
IGF type I receptor	95-135	IGF-I >> IGF-II	1×10^{9}	1×10^{8}
IGF type II receptor	230-270	IGF-II >> IGF-I	0.5×10^{7}	1×10^{9}

[a]From Conover, 1996

[b]MW of intact circulating form; on ligand blots, IGFBP-3 appears as a doublet at 40-43 kDa.

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Table 2

Percent amino acid homology of the IGFBPs across species[abc]

Bovine	Amino Acids[d]	Porcine	Ovine	Human	Mouse	Rat
IGFBP-1 (263)	234	NA[e]	NA	68 (259)	71 (272)	72 (272)
IGFBP-2 (53)	289	70 (23)	92 (317)	72 (328)	64 (305)	64 (305)
IGFBP-3 (291)	264	89 (266)	NA	82 (291)	78 (291)	80 (292)
IGFBP-4 (258)	237	88 (16)	98 (237)	97 (258)	89 (254)	89 (254)
IGFBP-5 (111)	252	99 (271)	NA	99 (272)	97 (254)	97 (254)
IGFBP-6 (122)	216	NA	NA	83 (240)	71 (238)	67 (226)

[a]Numbers in parenthesis indicate the amino acid length by which homology comparisons were made.
[b]European Bioinformatics Institute
[c]Swiss-Prot database
[d]Coverley and Baxter, 1997
[e]NA= not available

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CHAPTER III

Production of the Insulin-like Growth Factor Binding Proteins by Bovine Granulosa and Thecal Cells: Regulation by Metabolic Hormones

Abstract

To determine the hormonal effects on IGFBP production, granulosa and thecal cells from small (1 to 5 mm) and large (≥ 8 mm) bovine follicles, respectively, were collected, cultured for 2 days in 10% fetal calf serum, washed and cultured in serum-free medium for an additional 24 h with various hormone treatments, arranged in three experiments. Following treatment, cells were enumerated and medium was collected, concentrated 10-fold and subjected to ligand blotting. Experiment 1 revealed that ≥ 1.5 x 10^5 viable cells at plating were needed for maximal IGFBP production by granulosa and thecal cells. The major forms of IGFBPs produced were a 27-34-kDa IGFBP (IGFBP-2 and –5), and a 20- and 18-kDa IGFBP (IGFBP-4) by the granulosa cells and a 40-44-kDa IGFBP (IGFBP-3), 34-kDa IGFBP (IGFBP-2), 27-29-kDa IGFBP (IGFBP-5) and a 24- and 22-kDa IGFBP (IGFBP-4) by the thecal cells. In Experiment 2A, insulin stimulated production of the 27-29-kDa IGFBP (IGFBP-5) by thecal cells, and basic fibroblast growth factor (bFGF) inhibited the insulin-induced increase whereas only the combination of insulin and LH stimulated production of the 22-kDa IGFBP (IGFBP-4). Production of the 27-34-kDa IGFBP (IGFBP-2/5) by granulosa cells in Experiment 2B

was inhibited by insulin, with EGF and bFGF further enhancing insulin's inhibitory effect. In Experiment 3A, insulin enhanced production of the 27-29-kDa IGFBP (IGFBP-5) by thecal cells whereas glucagon blocked insulin's stimulatory effect. In contrast, insulin and glucagon synergistically diminished production of the 24-kDa IGFBP (IGFBP-4) whereas insulin with either glucagon or cortisol decreased production of the 22-kDa IGFBP (IGFBP-4) by the thecal cells. In Experiment 3B, production of the 27-34-kDa IGFBP (IGFBP-2/5) by granulosa cells was attenuated in the presence of cortisol with or without insulin and insulin with glucagon. Treatment with glucagon and cortisol decreased whereas insulin increased production of the 20- and 18-kDa IGFBPs (IGFBP-4), respectively, by granulosa cells. These results suggest that production of IGFBP-5 and -4 by thecal cells and IGFBP-2, -5, and -4 by granulosa cells are differentially affected by hormonal treatments.

Introduction

Folliculogenesis, or ovarian follicular growth, is an essential function of the reproductive system. During a normal estrous cycle, a network of hormonal events, which consist of peptide and steroid hormones, as well as growth factors, interact. Insulin-like growth factors-I and -II (IGF-I and -II) are two such examples of growth factors that play a role in modulating ovarian folliculogenesis. Folliculogenesis is an important factor that maintains overall production efficiency, it is crucial that the ovarian processes that controls follicular growth be understood in an effort to assess the reproductive system. Through an autocrine, paracrine, and endocrine fashion, the IGF's stimulate the growth and differentiation of granulosa and thecal cells as well as enhance

steroidogenesis (for review see Spicer and Echternkamp, 1995). Concentrations of IGF-I remain constant during the final stages of follicular growth (Stewart et al., 1996; de la Sota et al., 1996; Funston et al., 1996), indicating that the amount of IGF binding to its receptor and ultimately follicular growth, may be dictated by the insulin-like growth factor binding proteins (IGFBPs). Levels of IGFBPs in follicular fluid change dramatically during folliculogenesis. Specifically, IGFBP-2, a 22-kDa IGFBP (presumably IGFBP-4) and a 27-29-kDa IGFBP (presumably IGFBP-5) is predominately localized in large subordinate and small bovine follicles, with little to no activity of these three IGFBPs detected in the dominant follicles (de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996). Later during the cycle (approximately day 10) when the follicle is undergoing atresia, levels of IGFBP-2, -4, and -5 levels increase in the dominant follicle (Stewart et al., 1996). The varying pattern of these IGFBPs through the estrous cycle suggests that the low molecular weight IGFBP are hormonally regulated. In contrast, levels of IGFBP-3 remain constant during folliculogenesis (de la Sota et al., 1996; Funston et al., 1996; Stewart et al., 1996).

The hormones and growth factors that regulate follicular IGFBP production in cattle have not been determined. In cultured porcine granulosa cells, FSH inhibits, insulin stimulates, and EGF has no effect on IGFBP-2 production (Mondschein et al., 1990; Grimes and Hammond, 1992; Grimes et al., 1992). Both IGFBP-3 and -2 inhibit IGF-I induced steroidogenesis in bovine granulosa and thecal cells (Spicer et al., 1997; Spicer and Chamberlain, 1999). Thus, hormonal changes can alter the action of IGFs via changes in IGFBP. The scope of this study was to determine the hormonal regulation of IGFBP production by bovine granulosa and thecal cells, with specific

focus on the effects of insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), cortisol, and glucagon on IGFBP production.

Materials and Methods

Reagents and Hormones

The reagents used in this study include: Dulbecco's Modified Eagle's Medium (DMEM), Ham's F-12, insulin (bovine; 25.7 U/mg), fetal calf serum (FCS), cortisol, protease, collagenase, hyaluronidase, DNase, and acrylamide, all obtained from Sigma Chemical Company (St. Louis, MO); recombinant human basic fibroblast growth factor (bFGF) with carrier obtained from R & D Systems (Minneapolis, MN); recombinant human glucagon and recombinant human EGF obtained from Bachem California (Torrence, CA); bovine LH (USDA-bLH-B5: LH activity 2.1 x LH-SI U/mg; FSH activity < 1.0 % by weight) and ovine FSH (USDA-oFSH-19-SIAFP-I-2, FSH activity 94 x NIH-FSH-SI U/mg; LH activity .025 x NIH-LH-SI U/mg) obtained from the USDA, Animal Hormone Program (Beltsville, MD); bovine serum albumin (protease free) obtained from Intergen Co. (Purchase, NY); recombinant bovine IGF-II obtained from Monsanto (St. Louis, MO); 20% (w/v) sodium dodecyl sulfate solution (SDS) from Amresco (Solon, OH); and nitrocellulose transfer membrane (.45 µm pore size) from Midwest Scientific (St. Louis, MO).

Cell Culture

Ovaries from slaughtered pregnant and nonpregnant beef and dairy cattle were collected at a local commercial abattoir. The ovaries were placed in saline (.15 M NaCl) and transported to the laboratory (<120 min) where small follicles (1 to 5 mm) were aspirated using a 20 gauge needle (38.1 mm) and 3 mL syringe to collect granulosa cells as previously described (Langhout et al., 1991). The granulosa cells were washed 3 times in serum-free medium by centrifugation at 200 X g (for 5 to 7 min). The cells were then resuspended in medium containing 1 mg/mL collagenase and 0.01 mg/mL DNase to prevent clumping of the cells.

Thecal cells were collected from large follicles (≥ 8 mm) by dissection as previously described (Stewart et al., 1995). Briefly, after isolation, the theca interna was torn into small pieces and enzymatically digested for 1 h at 37° C on a rocking platform. Any tissue not digested after the incubation period was removed via filtration through a syringe filter holder with a metal screen (149 µm mesh; Gelman Sciences, Ann Arbor, MI). The thecal cells were then washed in serum-free medium by centrifugation (at 50 X g for 4 min) and resuspended in medium containing 1 mg/mL collagenase and 0.01 mg/mL DNase. The number of viable granulosa and thecal cells was determined by the trypan blue exclusion method and averaged 63.0% and 94.8%, respectively.

Medium consisted of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 containing 0.12 mM gentamicin, 20 mM glutamine, and 38.5 mM sodium bicarbonate. Approximately 3 x 10^5 viable cells (in 30-80 µl) were placed into each well of Falcon multiwell plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) containing 1 mL medium with 10% FCS, unless stated otherwise. Cells were cultured at 38.5° C with 95% air-5% CO_2 atmosphere, and the medium was changed

approximately every 24 h. Cell proliferation was monitored daily using phase contrast microscopy (Fig. 1A and 2A). During the first 2 days of incubation, cells were cultured in 10% FCS-containing medium, after which, the cells were washed twice with serum-free medium (0.5 mL), followed by 24 h hormonal treatments in 0.5 mL of serum-free medium containing 0.25 mg/mL of BSA (to minimize loss of the IGFBPs).

Experiment 1 was designed to determine whether cell density had any affect on IGFBP production by thecal (Experiment 1A) and granulosa cells (Experiment 1B). Thecal cells (Experiment 1A) from large follicles were plated at densities of .25, .5, 1.0, 1.5, 3.0, or 6.0 (x 10^5) cells/well in medium containing 10% FCS for 48 h. After 48 h, the media was replaced with serum-free medium containing insulin (100 ng/mL) and LH (100 ng/mL) and incubated for an additional 24 h. In Experiment 1B, granulosa cells from small follicles were plated at densities of .25, .5, 1.0, 1.5, and 3.0 (x 10^5) cells/ well in medium containing 10% FCS. After 48 h, the media was replaced with serum-free medium containing insulin (100 ng/mL) and FSH (50 ng/mL) and incubated for an additional 24 h.

Experiment 2A was designed to determine whether insulin, LH, EGF and/or bFGF affected IGFBP production by thecal cells. Thecal cells from large follicles (≥ 8 mm) were cultured for 48 h as described above. For the next 24 h the cells were cultured in serum-free medium containing no additions (control), insulin (100 ng/mL), LH (100 ng/mL), LH (100 ng/mL) plus insulin (100 ng/mL), EGF (10 ng/mL) plus insulin (100 ng/mL) or bFGF (10 ng/mL) plus insulin (100 ng/mL).

Experiment 2B was designed to determine whether insulin, FSH, EGF, and/or bFGF affected IGFBP production by granulosa cells. After the 48 h incubation period

in medium containing 10% FCS, granulosa cells from small (1 to 5 mm) follicles were incubated for another 24 h in serum-free medium containing no additions (control), insulin (100 ng/mL), FSH (50 ng/mL), FSH (50 ng/mL) plus insulin (100 ng/mL), EGF (10 ng/mL) plus insulin (100 ng/mL), or bFGF (10 ng/mL) plus insulin (100 ng/mL).

Experiment 3 was designed to determine whether insulin, cortisol and/or glucagon influenced IGFBP production by thecal (Experiment 3A) or granulosa (Experiment 3B) cells. After the 48 h incubation period in medium containing 10% FCS, thecal cells from large (≥8 mm) follicles and granulosa cells from small (1 to 5 mm) follicles were incubated for another 24 h containing no additions (control), insulin (100 ng/mL), cortisol (30 ng/mL), insulin (100 ng/mL) plus cortisol (30 ng/mL), glucagon (300 pg/mL), or insulin (100 ng/mL) plus glucagon (300 pg/mL).

Determination of numbers of granulosa and thecal cells

After the treatment period had elapsed, medium from each well was collected individually and frozen at (-20 °C) for later use. The number of granulosa and thecal cells were then determined using a Coulter counter (Model Zm; Coulter, Haileah, FL) as previously described (Langhout et al. 1991; Stewart et al., 1995). Briefly, the cells were washed twice with .5 mL of .15 M NaCl followed by the addition of .5 mL of trypsin (.25% wt/vol; for 20 min at 25°C). Following trypsin exposure, the cells were scraped from each well, diluted in .15 M NaCl, and enumerated.

Concentration of spent medium

To concentrate the IGFBPs, the media samples collected after cells were exposed to treatment for 24 h were ultrafiltrated using Centricon concentrators with a

molecular weight (MW) limit of 3,000 (Amicon, Inc., Beverly, MA). The spent medium was concentrated approximately 10-fold. Briefly, 400 µL of the spent media was placed inside the sample reservoir of the concentrator and centrifuged at 5322 X g for approximately 80 min. After centrifugation, the filtrate vial was discarded, the sample reservoir was inverted and re-centrifuged for 5 min at 591 X g to transfer the retentate into the retentate vial. Final volumes, ranging from 29 to 70 µL were measured to record the concentration factor of the sample; this value was used to correct data.

Gel electrophoresis of the IGFBPs

The concentrated media samples were assessed for IGFBP activity based on molecular weight using one-dimensional, reducing, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Laemmli, 1970; Stewart et al., 1996). Briefly, 12.5 µL of the concentrated sample was mixed with 12.5 µL of Laemmli sample buffer (BIORAD, Hercules, CA). After heat treatment (3 min at 100 °C) to denature the proteins, samples were centrifuged at 4657 X g for 3 min and individually added to the 12-lane SDS-PAGE. Twenty-five µL of a wide range color marker (MW 6,500 to 205,000, Sigma, St. Louis, MO) and a mixture of 4 uL bovine follicular fluid with 21 µL of the sample buffer were included to serve as controls. The samples were electrophoresed overnight (approximately 18 to 20 h) at constant current (25 to 35 amps) and varying voltage. Following electrophoresis, the gels were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) for 2.5 to 3.0 h. Each nitrocellulose paper was then labeled with ^{125}I-IGF-II (approximately 15,000 cpm/0.1 mL; total volume=12 mL) and placed on a rocking

platform at 4° C overnight. The next day, the blots were washed in a Tris-buffered saline (TBS) with 0.1% Tween followed with additional washings with only the TBS. The nitrocellulose blots were then dried and placed on X-ray film for approximately 14 d at -80° C. At the end of the 14-d period, X-ray films were developed and individual bands were densitometrically analyzed using Molecular Analyist (BIO-RAD, Hercules, CA).

Statistical Analysis

Data are presented as the least squares means ± SE. Each experiment was replicated 2 to 3 times with different pools of granulosa cells, each collected from 20 to 60 ovaries, whereas each pool of thecal cells was obtained from 6 to 8 follicles from 4 to 10 cows. For each experiment, treatments were applied in triplicate culture wells. Using the general linear model procedures of SAS, treatment effects and interactions were determined. Specifically, main effects (treatments and replicates) and interactions on dependent variables (i.e., IGFBP production) were analyzed. IGFBP production, which was corrected for final cell numbers and the concentration factor, was expressed as arbitrary densitometric units per 10^5 cells per 24 h. Specific differences in IGFBP production between treatments were determined using PDIFF (SAS, 1988) if significant main effects were observed.

Results

Experiment 1A: Effect of thecal cell plating density on IGFBP production

Thecal cells produced at least five forms of IGFBP: a 42-44-kDa (IGFBP-3), a 34-kDa (IGFBP-2), a 27-29-kDa IGFBP (IGFBP-5), a 24-kDa IGFBP (IGFBP-4) and a 22-kDa IGFBP (IGFBP-4). IGFBP-4 (24-kDa) and IGFBP-5 were the major IGFBPs produced (Fig. 1B). Plating density significantly ($p < .05$) affected production of all IGFBPs (Fig. 3). Increasing thecal cell plating density from 1 to 3 x 10^5 cells/well increased ($p < .05$) production of all the IGFBPs on a per cell basis. A plating density of 6.0 x 10^5 cells/well had no further influence on production of IGFBP-3, –2, -5, and -4 (22-kDa) from that observed with 3.0 x 10^5 cells/well. A similar plateau effect was demonstrated with the 24-kDa IGFBP (IGFBP-4) subsequent to a plating density of 1.5 x 10^5 cells/well (Fig. 3D).

After 3 d in culture, thecal cell numbers averaged .48, .90, 1.64, 2.15, 3.57, 5.37, ± .05 x 10^5 cells/well for .25, .5, 1.0, 1.5, 3.0, and 6.0 x 10^5 cells plated per well. Increasing plating density from 0.25 x 10^5 to 6.0 x 10^5 cells/well resulted in proportional increases ($p < .05$) in thecal cell numbers after 3 d in culture.

Experiment 1B: Effect of granulosa cell density on IGFBP production

Granulosa cells produced at least three forms of IGFBPs; a 27-34-kDa IGFBP (IGFBP-2 and -5), an 18-and 20-kDa IGFBP (IGFBP-4); IGFBP-2/5 was clearly the predominant IGFBP produced (Fig. 2B). Plating density affected granulosa cell production of IGFBP-2/5 ($p < .05$) and IGFBP-4 (20-kDa and 18-kDa, $p < .05$). Increasing granulosa cell plating density from .25 x 10^5 to 1.5 x 10^5 cells/well increased production (on a per cell basis) of IGFBP-2/5 and IGFBP-4 (20-kDa and 18-kDa, Fig. 4). However, increasing plating densities from 1.5 to 3.0 x 10^5 cells/well did not ($p >$

.05) further enhance production of IGFBP-2/5 and the 18-kDa IGFBP (IGFBP-4, Fig. 4).

After 3 d in culture, granulosa cell numbers averaged 0.45, 1.13, 2.04, 2.49, and 3.35 ± 0.10 cells/well for 0.25, 0.50, 1.0, 1.5, and 3.0 x 10^5 cells plated per well, respectively. Increasing plating density from .25 x 10^5 to 3.0 x 10^5 cells/well, resulted in proportional increases ($p < .05$) in granulosa cell numbers after 3 d in culture.

Experiment 2A: Effect of insulin, LH, EGF, and bFGF on thecal cell IGFBP production

As in Experiment 1A, the two major IGFBPs produced by thecal cells was the 27-29-kDa (IGFBP-5) and 24-kDa (IGFBP-4) IGFBP. Treatment had no effect ($p > .10$) on IGFBP-2 production, but tended ($p < .10$) to affect production of IGFBP-3 and the 24-kDa IGFBP (IGFBP-4, Table 3). Insulin and insulin plus LH tended ($p < .10$) to increase production of the 24-kDa IGFBP (IGFBP-4) whereas the combined treatments of insulin with either LH, EGF or bFGF tended ($p < .10$) to increase production of IGFBP-3. In contrast, significant treatment effects on IGFBP-5 and the 22-kDa IGFBP (IGFBP-4) were observed (Fig. 3). Insulin increased ($p < .05$) production of IGFBP-5, the predominant IGFBP in this experiment, whereas LH had no affect ($p > .10$) on basal or insulin-stimulated IGFBP-5 production. EGF had no effect whereas bFGF blocked the insulin-induced increase in the production of the IGFBP-5 (Fig. 5A). Insulin plus LH increased production of the 22-kDa IGFBP (IGFBP-4) when compared to the control ($p < .05$, Fig. 5B). Neither LH or insulin alone significantly affected production of the 22-kDa IGFBP (IGFBP-4).

The influence of the treatments on thecal cell numbers is depicted in Table 3. Insulin ($p < .05$) increased the number of thecal cells, but LH had no affect on basal or insulin-induced thecal cell numbers. In contrast, both EGF and bFGF further increased ($p < .05$) the insulin-stimulated cell numbers.

Experiment 2B: Effect of FSH, insulin, EGF, and bFGF by granulosa cell IGFBP production

As in Experiment 1B, the major IGFBP produced by the granulosa cells was the 27-34-kDa IGFBP (IGFBP-2/5). Treatments had no effect ($p > .10$) on the 18-kDa (IGFBP-4) and 20-kDa IGFBP (IGFBP-4, Table 4). In contrast, treatment effects were observed on IGFBP-2/5 ($p < .05$). Treatment with insulin alone and FSH plus insulin decreased production of IGFBP-2/5. However, the addition of FSH alone had no effect (Fig. 6A). Both EGF and bFGF further augmented the inhibitory effect of insulin on production of IGFBP-2/5 (Fig. 6B).

Table 4 shows the effects of the treatments on granulosa cell numbers. Insulin and insulin plus FSH increased ($p < .05$) granulosa cell numbers similarly. EGF and bFGF further increased ($p < .05$) insulin-induced granulosa cell numbers. However, FSH had no effect ($p > .10$) on basal or insulin-induced cell numbers (Table 4).

Experiment 3A: Effect of insulin, glucagon and cortisol on thecal cell IGFBP production

The predominant IGFBP produced by thecal cells of these experiments was the 24-kDa IGFBP (IGFBP-4). Treatments had no effect ($p > .10$) on IGFBP-3 production by the thecal cells whereas a trend ($p < .10$) for a treatment effect was observed with IGFBP-2 (Table 5). Production of IGFBP-2 was greater ($p < .10$) in cells treated with

insulin than those treated with either glucagon or cortisol (Table 5). Insulin tended ($p < .10$) to increase IGFBP-2 production in the presence of glucagon. In contrast, treatment effects were observed on IGFBP-5 ($p < .05$), the 24-kDa (IGFBP-4, $p < .05$), and 22-kDa (IGFBP-4, $p < .05$) IGFBP (Fig. 7). Production of IGFBP-5 (Fig. 7A) was stimulated ($p < .05$) by insulin alone whereas other singular treatments (i.e., glucagon and cortisol) were without effect ($p > .05$). Glucagon inhibited insulin-stimulated production of IGFBP-5 whereas cortisol had no effect (Fig. 7). Production of the 24-kDa IGFBP (IGFBP-4) was inhibited by the combination of insulin and glucagon, whereas other treatments were without effect (Fig. 7B). Likewise, production of the 22-kDa IGFBP (IGFBP-4, Fig. 7C) was inhibited by insulin plus glucagon, cortisol alone, and cortisol plus insulin.

Table 5 shows the effects of the treatments on cell numbers. Insulin increased ($p < .05$) the number of thecal cells, whereas glucagon and cortisol had no effect ($p > .10$) on basal or insulin-induced cell numbers (Table 5).

Experiment 3B: Effect of insulin, glucagon, and cortisol on granulosa cell IGFBP production

The major IGFBP produced by granulosa cells in this experiment was the 27-34-kDa IGFBP (IGFBP-2/5). Treatment effects were observed on IGFBP-2/5, the 20-kDa (IGFBP-4), and 18-kDa (IGFBP-4) IGFBPs ($p < .05$, Fig. 8). Cortisol, insulin plus glucagon, and insulin plus cortisol inhibited ($p < .05$) production of IGFBP-2/5; singular treatment of insulin or glucagon had no effect (Fig. 8A). In contrast, production of the 20-kDa (IGFBP-4) was inhibited ($p < .05$) by singular treatment of cortisol or glucagon and by the combination of insulin and glucagon; insulin with or without cortisol had no

effect ($p > .10$, Fig. 8B). The 18-kDa IGFBP (IGFBP-4) was stimulated ($p < .05$) by insulin treatments alone, while all other treatments were without effect ($p > .10$, Fig. 8C).

Granulosa cell numbers averaged 1.63, 1.61, 1.19, 1.04, 0.94, and $0.84 \pm .16 \times 10^5$ cells/well for control, insulin, glucagon, cortisol, insulin plus glucagon and insulin plus cortisol treatment groups, respectively. Insulin increased ($P < .05$) granulosa cell numbers whereas glucagon or cortisol had no effect ($P > .10$) on basal or insulin-induced cell numbers.

Discussion

Results of the present study revealed that 1) a 42-44-kDa (IGFBP-3), 34-kDa (IGFBP-2), 27-29-kDa, 24-kDa and 22-kDa IGFBP was produced by thecal cell cultures (Fig. 1B) while a 27-34-kDa, 20-kDa and 18-kDa IGFBP was produced by granulosa cell cultures (Fig. 2B), 2) insulin enhanced production of the 27-29-kDa and 18-kDa IGFBP by thecal (Table 6) and granulosa cells (Table 7), respectively, 3) alone LH or FSH had no affect on thecal (Table 6) or granulosa (Table 7) cell IGFBP production, 4) EGF and bFGF decreased production of the 27-34-kDa IGFBP by granulosa cells (Table 7), 5) alone glucagon had no effect on thecal or granulosa cell IGFBP production, but decreased IGFBP production in the presence of insulin (Table 6 and 7) and , 6) alone cortisol decreased production of the 22-kDa IGFBP by thecal cells and the 27-34-kDa and 20-kDa IGFBP by granulosa cells (Table 6 and 7).

Based on immunoblotting techniques from other laboratories, the 42-44-kDa is likely IGFBP-3, the 34-kDa is likely IGFBP-2, the 27-29-kDa is likely IGFBP-5 and the

24-kDa is likely IGFBP-4 (Stanko et al., 1994; de la Sota et al., 1996; Funston et al., 1996). While the identity of the 22-, -20, and 18-kDa bands remains undetermined, results from human follicular fluid (Cataldo and Giudice, 1992a,b) and rat granulosa cell conditioned medium (Liu et al., 1993) suggest that these IGFBPs may represent different glycosylated forms of IGFBP-4. In addition, 27-34-kDa by the granulosa cells is likely to be a composite of two IGFBPs, IGFBP-2 and -5. Because the bands identifying the two different IGFBPs were indistinguishable on the ligand blots, the IGFBPs were scanned and analyzed as one.

The IGFBPs most influenced by hormone treatments were IGFBP-5 and the 24-kDa (IGFBP-4) IGFBPs by the thecal cells and IGFBP-5 and -2 by the granulosa cells, the major IGFBP produced by each cell type (Table 6 and 7). In contrast, IGFBP-3 and IGFBP-2, minor IGFBPs produced by the thecal cells, were not influenced by any hormone regime. The lack of response of IGFBP-2 as well as IGFBP-3 to hormone treatments in cultured thecal cells in the current study may stem from the low expression of these mRNA in the thecal cells. In support of this suggestion, in situ hybridization experiments in sheep follicles indicated that the IGFBP-2 mRNA is primarily expressed in granulosa cells whereas, IGFBP-4 and -5 mRNA are expressed in thecal cells in healthy ovine follicles (Besnard et al., 1996) and that IGFBP-3 mRNA is low or undetectable in ovine (Perks and Wathes, 1996) and bovine (Kirby et al., 1996; Yuan et al., 1998) follicles and ovaries. However, both IGFBP-2 and -5 are secreted by cultured ovine granulosa cells (Armstrong et al., 1996; Monget et al., 1998). In the rat, thecal, but not granulosa cells contain IGFBP-2 mRNA whereas the converse is true for the mouse (Ricciarelli et al., 1991; Erickson et al., 1995; Adashi et al., 1997).

In both the rat and mouse, IGFBP-4 mRNA is found in mainly granulosa cells with some expression in thecal cells (Nakatani et al., 1991; Erickson et al., 1992a; Adashi et al., 1997), whereas IGFBP-5 mRNA is found in granulosa but not thecal cells (Erickson et al., 1992b; Adashi et al., 1997). Thus, species differences may exist in terms of which cell type produce a particular IGFBP. Similar to previous results in rats, bovine thecal cells, but not granulosa cells produced IGFBP-3 in the present study (Liu et al., 1993; Nakatani et al., 1991; Ricciarelli et al., 1991; 1992). Although IGFBP-3 is the primary IGFBP found in bovine follicular fluid (Stewart et al., 1996; Funston et al., 1996; Echternkamp et al., 1994), the levels of IGFBP-3 in follicular fluid reflect those found in the systemic circulation of cattle (Stewart et al., 1996), suggesting that the majority of IGFBP-3 found in follicular fluid may be transuded from blood. Lack of hormone responsiveness of IGFBP-3 production by thecal cells as well as low amounts produced, support this conclusion.

Similar to previous research, EGF stimulated mitogenic activity of insulin in cultured granulosa (Savion et al., 1981; May et al., 1988) and thecal cells (May et al., 1992; Vernon and Spicer, 1994; Spicer and Stewart, 1996) in the present study. EGF inhibits steroidogenesis of both cell types (Langhout et al., 1991; Spicer and Stewart, 1996). EGF plus insulin inhibited production of the IGFBP-2/5 by the granulosa cells, but had no effect on thecal cell IGFBP production. Similar to our finding with thecal cells, EGF, or its homologue, TGFα, had no effect on IGFBP-2 and -3 production by cultured porcine granulosa cells (Mondschein et al., 1990), IGFBP-4 and -5 mRNA levels in whole ovarian homogenates of rats (Chamoun et al., 1999), IGFBP-2 production by cultured human intestinal epithelial cells (Oguchi et al., 1993) and

vascular smooth muscle cells (Bourner et al., 1992; Cohick and Clemmons, 1993), and IGFBP-3 production by cultured human intestinal (Oguchi et al., 1993) or retinal pigment-epithelial cells (Feldman and Randolph, 1994). However, EGF increased IGFBP-3 production by cultured rat astroblasts (Loret et al., 1991) and decreased IGFBP-3 production in human cervical epithelial cells (Andreatta-Van Leyen et al, 1994) and keratinocytes (Wraight and Werther, 1995), as well as mouse embryo fibroblasts (Villaudy et al., 1991). The lack of consistency resulting from EGF treatment also applies to IGFBP-4. Our observation that IGFBP-4 production by thecal or granulosa cells was not affected by EGF treatment agrees with data from cultured human fibroblasts (Conover et al., 1993b), but disagrees with data from cultured human intestinal epithelial cells (Oguchi et al., 1993). Consistent with our findings in granulosa cells, EGF decreased IGFBP-5 gene expression by rabbit proximal tubular cells (Yap et al., 1997). Collectively, these results suggest that the regulation of IGFBP production by EGF treatment differs with cell type. The physiologic consequences of the inhibitory effect of EGF on ovarian IGFBP synthesis could allow for more available IGF during early follicle growth.

Like EGF, bFGF stimulated proliferation of thecal and granulosa cells in the present and previous studies (Gospodarowicz and Bialecki, 1978; 1979; Gospodarowicz et al., 1985; Wandji et al., 1992; Vernon and Spicer, 1994; Spicer and Stewart, 1996); bFGF also inhibits steroidogenesis of both cell types (Vernon and Spicer, 1994; Spicer and Stewart, 1996). In the present study, bFGF decreased production of IGFBP-2/5 by the granulosa cells and IGFBP-5 and the 22-kDa IGFBP (IGFBP-4) by the thecal cells. Previous research indicates that the influence of bFGF, like EGF, is dependent on the

particular IGFBP evaluated and cell type used. For example, IGFBP-2 production was not influenced by bFGF treatment in cultured vascular smooth muscle cells (Bourner et al., 1992; Cohick and Clemmons, 1993; Cohick et al., 1995) but was increased in human cultured neuroblastoma cells (Babajko et al., 1997). However, treatment of bFGF to fetal rat osteoblasts (Canalis et al., 1993; Ganalis and Gabbitas, 1995) and rat whole ovarian homogenates (Chamoun et al., 1999), decreased IGFBP-5 protein as well as its mRNA which agrees with the current research. Also in agreement with the present study, bFGF had no effect of IGFBP-4 and -5 mRNA levels in rat whole ovarian homogenates (Chamoun et al., 1999) and rat granulosa cells (Choe et al., 1997). In comparison, IGFBP-3 production by thecal cells was not effected by bFGF in our current work. Studies using mouse fibroblasts (Blat et al., 1989), chick embryo fibroblasts (Blat et al., 1989), human keratinocytes (Wraight and Werther, 1995), and rat astroblasts (Loret et al., 1991) found a decrease in IGFBP-3 levels with bFGF treatments. Similar to what was suggested for EGF, bFGF may enhance follicular growth by increasing bioavailable IGF-I via decreased IGFBP production.

Similar to previous studies using porcine (Mondschein et al., 1990; Grimes et al., 1992), rat (Liu et al., 1993; Fielder et al., 1993; Choi et al., 1996), and polycystic human (San Roman and Magoffin, 1992) granulosa cells, FSH decreased production of IGFBP-2/5 by bovine granulosa cells when co-incubated with insulin. In contrast, FSH treatment was ineffective at modulating IGFBP-2 production by cultured human (Cataldo et al., 1993) and ovine (Armstrong et al.,1996) granulosa cells. Echternkamp et al. (1994) likewise indicated that administration of exogenous FSH to cyclic cows had no effect on the low molecular IGFBPs (i.e., IGFBP-2, -4, and -5) in large ($\geq$ 8 mm)

estrogen-inactive follicles, but decreased their amounts in large estrogen-active follicles. Erickson et al. (1994) and Piferrer et al. (1997) showed that low doses of FSH (≤ 3 ng/mL) increased IGFBP-4 production whereas high doses (≥ 10 ng/mL) inhibited IGFBP-4 levels by cultured rat granulosa cells. Similar biphasic effect of FSH was observed on IGFBP-5 production by cultured rat granulosa cells (Adashi et al., 1990a; 1991; Fielder et al., 1993). The negative effect of the high doses of FSH was due to FSH stimulating proteolysis of IGFBP-4 and -5 (Liu et al., 1993; Fielder et al., 1993; Piferrer et al., 1997) as well as decreasing IGFBP-4 mRNA levels (Erickson et al., 1994; Choi et al., 1996). Thus, the inconsistent effects of FSH among studies may be due to the variable concentration of FSH used among experiments, the physiological status of the follicle, and/or species differences. If indeed low doses of FSH stimulate, and high doses decrease production of the IGFBP-2, -4, and/or -5, then this would suggest that IGFBP-2, -4, and -5 may be developmentally regulated during the estrous cycle.

In contrast to FSH, LH did not significantly affect production of any of the IGFBPs by bovine thecal cells of the present study. Voutilainen et al. (1996) reported that LH treatment increased IGFBP-5 mRNA levels in cultured thecal cells. Giudice et al. (1991) showed that the addition of hCG to cultured human luteinized granulosa cells increased IGFBP-4 production 6- to 8-fold above the controls. Moreover, LH treatment caused a dose- and time-dependent increase of IGFBP-4 production in cultured ovine thecal cells (Armstrong et al., 1996) and IGFBP-4 mRNA in cultured bovine thecal cells (Armstrong et al., 1996). Previously, LH had no effect on IGFBP-2 production in human granulosa cells (Giudice et al., 1991; Cataldo et al., 1993), rat thecal cells

(Erickson et al., 1995), and ovine thecal cells (Armstrong et al., 1996). Similarly, LH had no effect on IGFBP-2 and -3 mRNA levels in cultured human thecal cells (Voutilainen et al., 1996) and granulosa cells (Giudice et al., 1991). LH also had no effect on IGFBP-4 production by rat thecal cells (Erickson et al., 1995). In contrast, LH decreased IGFBP-3 production by human granulosa cells (San Roman and Magoffin, 1992). These studies suggest that the influence of LH on IGFBP production may be species dependent or dependent on whether thecal or granulosa cells have luteinized in vitro.

For the first time, we have found that cortisol inhibited production of IGFBP-2/5 and the 20-kDa (IGFBP-4) IGFBP by the granulosa cells with little or no effect on IGFBP production by the thecal cells. Of the hormones tested, cortisol was the most influential on granulosa cell IGFBP production, inhibiting IGFBP production in the presence and absence of insulin. In comparison, cortisol decreased production of the 24-kDa IGFBP (IGFBP-4) by the thecal cells in the absence of insulin, but had no effect on production of the other IGFBPs (i.e., IGFBP-2, -3, -5). Similarly, IGFBP-4 production was decreased with dexamethasone in cultured human osteoblast cells (Durham et al., 1994). In contrast, IGFBP-2 and -4 production increased with dexamethasone and hydrocortisone application in cultured mouse pancreatic β-cells and rabbit proximal tubular cells, respectively (Katz et al.,1997; Yap et al.,1997) whereas dexamethasone had no effect on IGFBP-4 production by cultured human fibroblast cells (Conover et al., 1993a). Likewise, IGFBP-3 production was also variable with cortisol treatments, depending on the cell type. For instance, dexamethasone had no influence on IGFBP-3 production by human keratinocytes (Wraight and Werther, 1995) but

decreased production by cultured human fibroblasts (Camacho-Hubner et al., 1992). In vivo, IGFBP-3 levels were stimulated by dexamethasone in healthy males (Miell et al., 1993; 1994). Treatment of cortisol inhibited IGFBP-5 production by cultured fetal rat osteoblast cells (Gabbitas et al., 1996) and human fibroblasts (Camacho-Hubner et al., 1992). Thus, the specific effect of cortisol on a particular IGFBP depends on the cell-type. Because IGFBP production is associated with follicular atresia, the cortisol-induced decrease in IGFBP production in granulosa and thecal cells may help maintain or further stimulate follicle growth. In the cow, cortisol increases both insulin- and IGF-I-induced progesterone and androstenedione production in cultured thecal cells (Spicer and Chamberlain, 1998). Collectively, these results imply that cortisol may be involved with the maintenance or growth of ovarian follicles in cattle.

Insulin consistently increased IGFBP-5 and the 18-kDa IGFBP (IGFBP-4) by thecal and granulosa cells, respectively. Similarly, insulin increased production of IGFBP-4 by cultured human fibroblast cells (Camacho-Hubner et al., 1992). Insulin's effect on IGFBP-4 may be dose-dependent because high insulin doses ($\geq$ 100 ng/mL) increased IGFBP-4 by human endometrial stromal cells (Irwin et al., 1995) and rat hepatocytes (Scharf et al., 1996) and low doses of insulin ($\leq$ 10 ng/mL) did not affect IGFBP-4 levels in human endometrial stromal (Irwin et al., 1995) or neuroblastoma (Babajko et al., 1997) cells. In contrast to IGFBP-4, high doses of insulin appear to decrease IGFBP-2 production by rat intestinal epithelial cells (Guo et al., 1995) and rat hepatocytes (Scharf et al., 1996). Insulin treatments did not have any significant effect on IGFBP-5 production by cultured human osteoblast cells (Conover and Kiefer, 1993) and fibroblasts (Camacho-Hubner et al., 1992). Similar to what was found for thecal

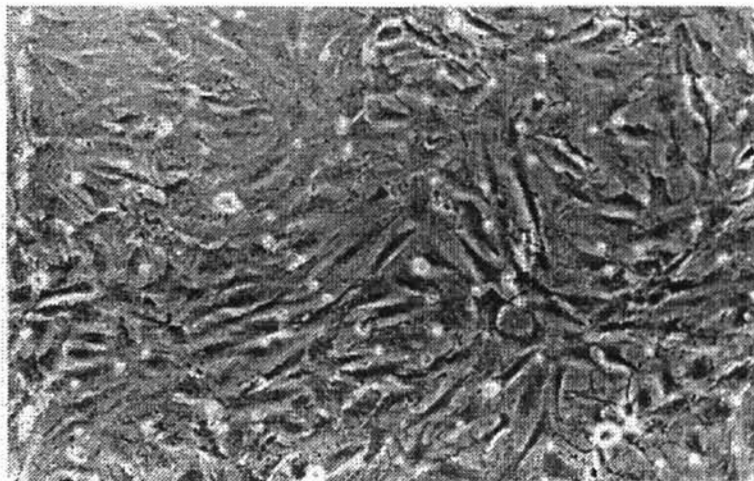
cells of the present study, production of IGFBP-3 protein or mRNA by cultured human retinal pigment epithelial cells (Feldman and Randolph, 1994), as well as IGFBP-3 protein by cultured human fibroblasts (Camacho-Hubner et al., 1992) and granulosa cells (Cataldo et al., 1993) were not affected by insulin treatments. The discrepancies observed with the current study versus previous research suggests that the specific effect of insulin on production of a particular IGFBP may be dependent on cell-type and concentration of insulin used.

For the first time, an effect of glucagon within the ovary has been demonstrated. Specifically, glucagon blocked the stimulatory effect of insulin on production of IGFBP-5 by thecal cells and the 18-kDa (IGFBP-4) by granulosa cells. Alone, glucagon had little or no effect. In the presence of insulin, glucagon also decreased production of the 24- and 22-kDa IGFBP (IGFBP-4) by thecal cells, and IGFBP-2/5 by granulosa cells. Although Denver and Nicoll (1994) found no significant effect of glucagon on IGFBP-2 and -4 production by primary rat hepatocytes, IGFBP-1 levels were decreased. GH deficient patients administered glucagon experienced increased serum IGFBP-1 levels (Hilding et al., 1993). Serum insulin and glucose levels transiently increased as well, but decreased following the IGFBP-1 increase. The decrease in insulin would suggest that glucagon overcomes insulin stimulation of IGFBP-1; an observation we also demonstrated in the current research. Thus, glucagon appears to exert an inhibitory effect on bovine ovarian IGFBP production in vitro.

Based on the current and previous results, the mechanism by which IGFBP production is regulated is likely through a network of hormones. Because IGFBP activity decreases during follicular growth and increases during follicular atresia, the

changes in hormone concentrations during the estrous cycle are likely changing IGFBP production. This in turn would decrease IGFBP activity and result in an increase in bioavailable IGF. Because IGF-I and -II remains relatively constant throughout follicle growth, fluctuations in IGFBP activity during the estrous cycle appear to regulate the bioavailability of IGF.

A



B

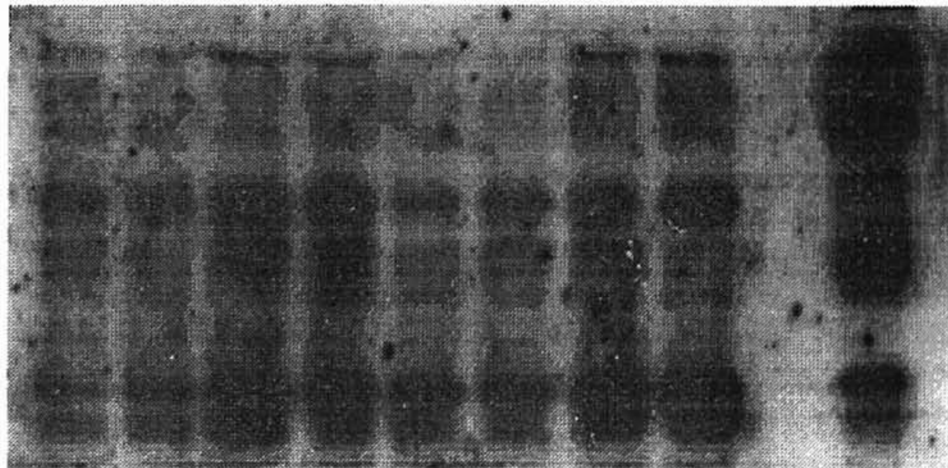
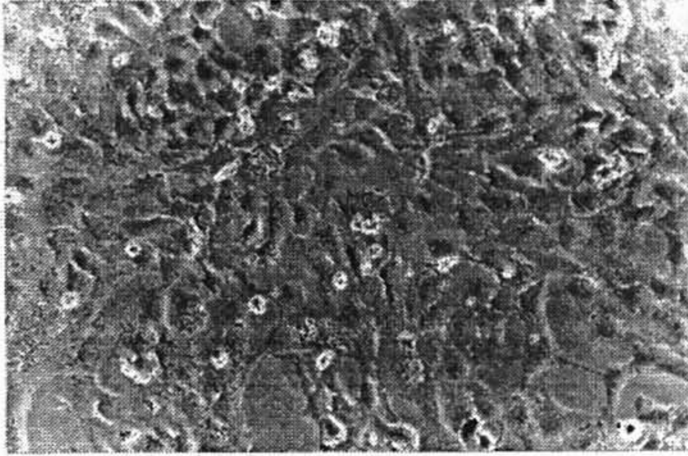


Figure 1: Panel A: representative example of cultured thecal cells under phase contrast microscopy. Panel B: representative ligand blot of thecal cell IGFBP production.

A



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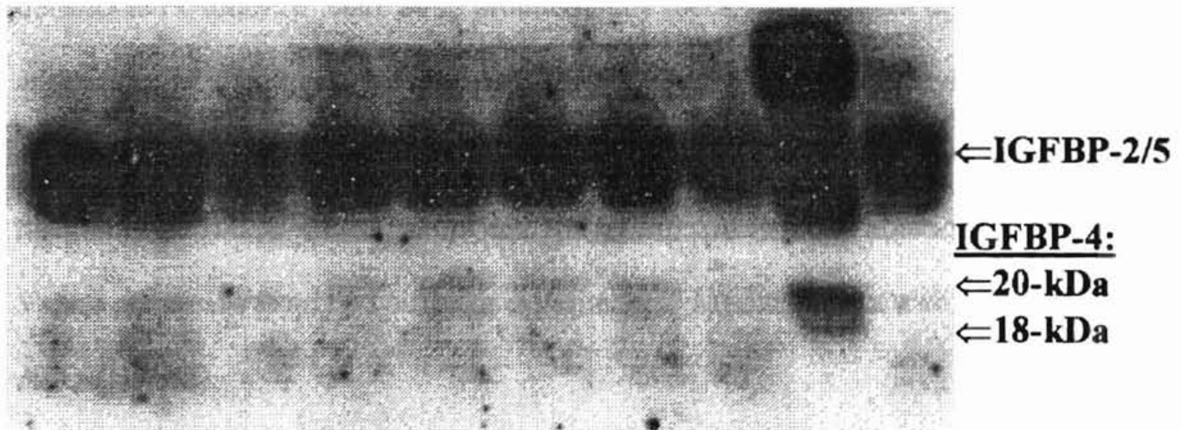
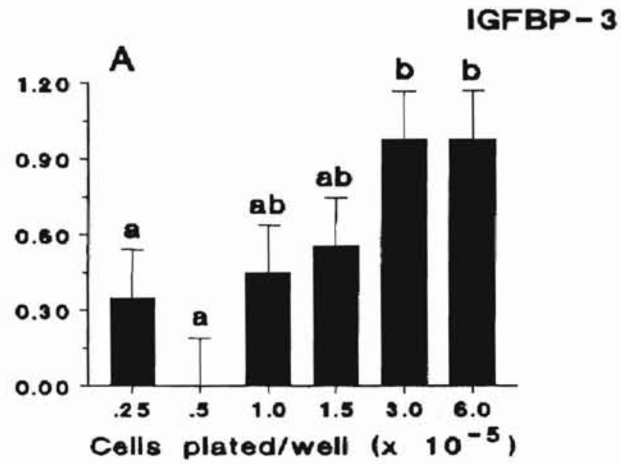


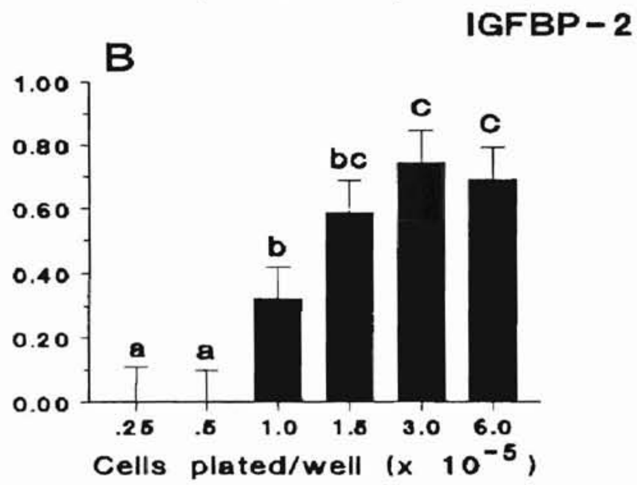
Figure 2: Panel A: representative example of cultured granulosa cells under phase contrast microscopy. Panel B: representative ligand blot of granulosa cell IGFBP production.

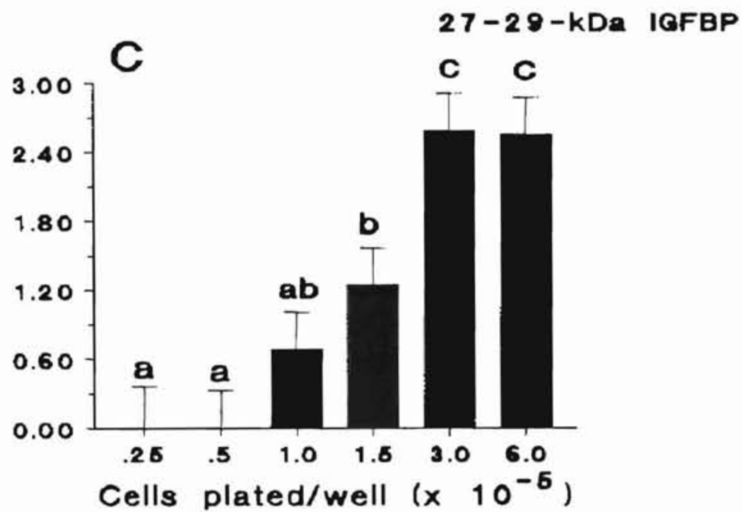
Figure 3. Effect of thecal cell density on IGFBP-3 (A), IGFBP-2 (B), the 27-29-kDa (IGFBP-5, C), 24-kDa (IGFBP-4, D), and 22-kDa (IGFBP-4, E) IGFBP (arbitrary densitometric units/10^5 cells/24 h) production by thecal cells. Thecal cells were plated at densities of .25, .5, 1.0, 1.5, 3.0, or 6.0 (x 10^5) and cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated with serum-free medium with 100 ng/mL of insulin and 100 ng/mL of LH for an additional 24 h. Values are means from two different experiments. Means without a common letter differ ($p < .05$).

Thecal cells

Binding activity (arbitrary units/10^5 cells/24 h)

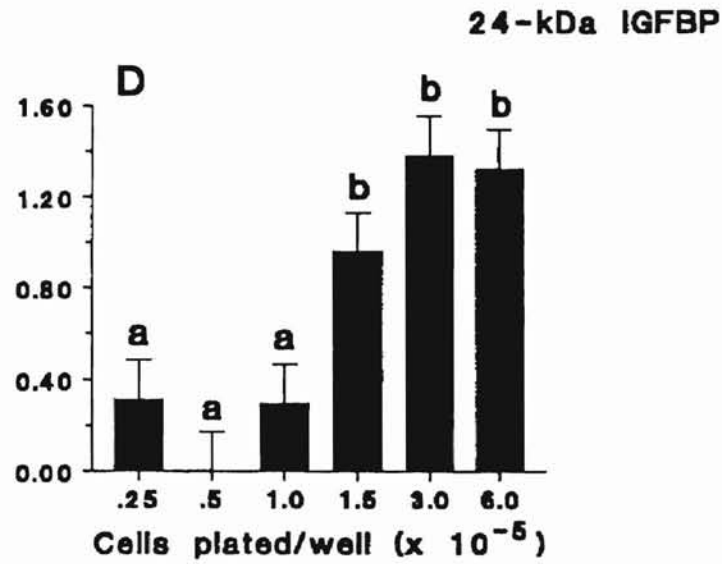






Thecal cells

Binding activity (arbitrary units/10^5 cells/24 h)



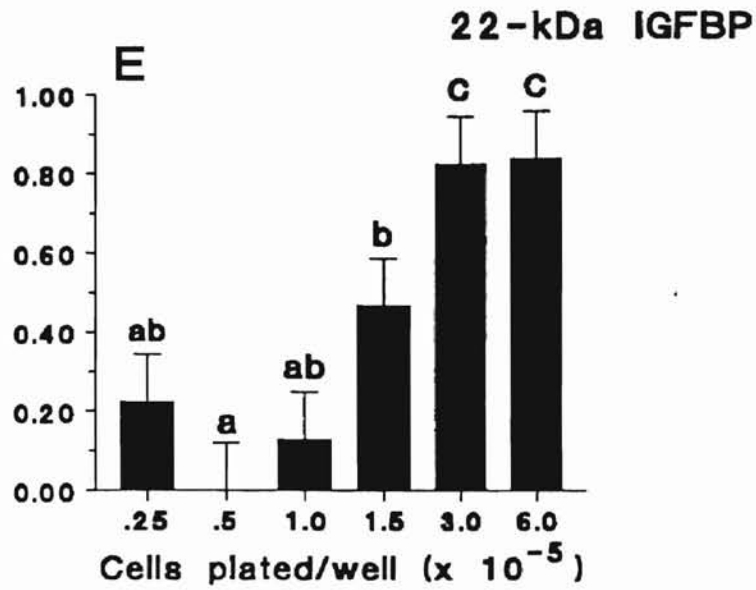
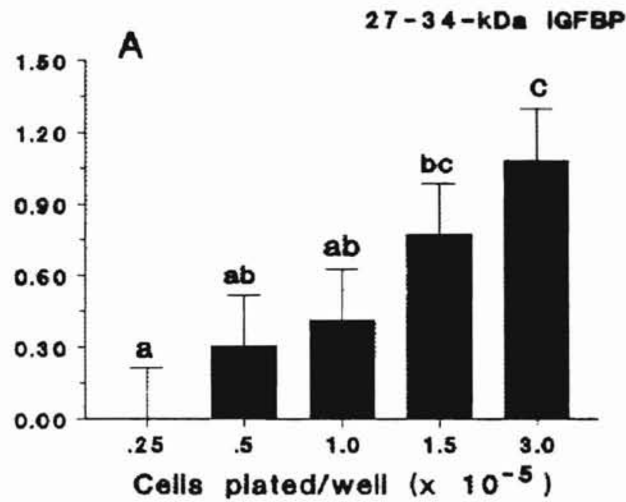
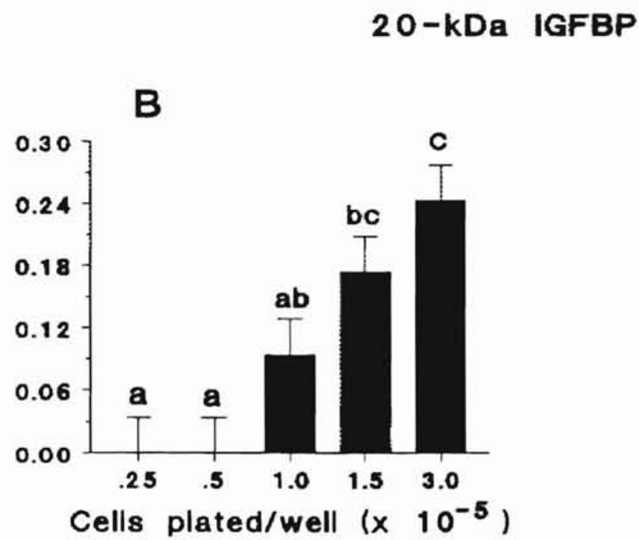


Figure 4. Effect of cell density on the 27-34-kDa (IGFBP-2/5, A), 20-kDa (IGFBP-4, B), and 18-kDa (IGFBP-4,C) IGFBP (arbitrary densitometric units/10^5 cells/24 h) production by granulosa cells. Granulosa cells were plated at densities of 0.25, 0.5, 1.0, 1.5 or 3.0 (x 10^5) and cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated with serum-free medium with 100 ng/mL of insulin and 50 ng/mL of FSH for an additional 24 h. Values are means from three different experiments. Means without a common letter differ ($p < .05$).

Granulosa cells

Binding activity (arbitrary units/10^5 cells/24 h)





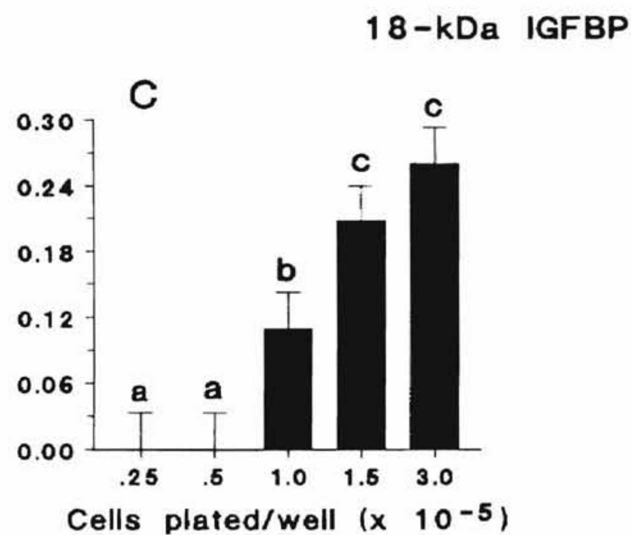
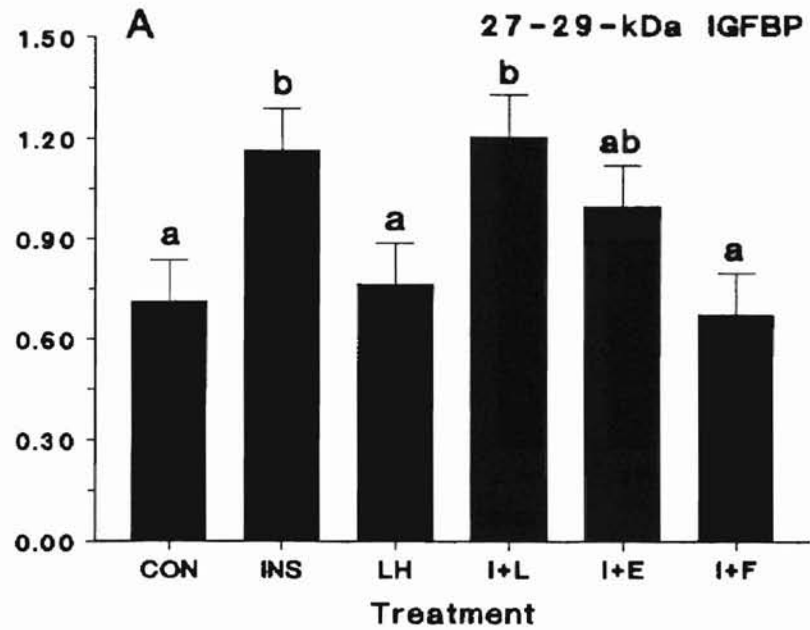


Figure 5. Effect of insulin, LH, EGF, and bFGF on the 27-29-kDa (IGFBP-5, A) and 22-kDa (IGFBP-4, B) IGFBP production (arbitrary densitometric units/10^5 cells/24 h) by thecal cells from large follicles (≥ 8 mm). Thecal cells were cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin (INS), 100 ng/mL of LH (LH), 100 ng/mL of insulin plus 100 ng/mL of LH (I + L), 10 ng/mL of EGF plus 100 ng/mL of insulin (I + E), 10 ng/mL of bFGF plus 100 ng/mL of insulin (I + F) or no additions (CON) for and additional 24 h. Values are means from three separate experiments. Means without a common letter differ ($p < .05$).

Thecal cells

Binding activity (arbitrary units/10^5 cells)



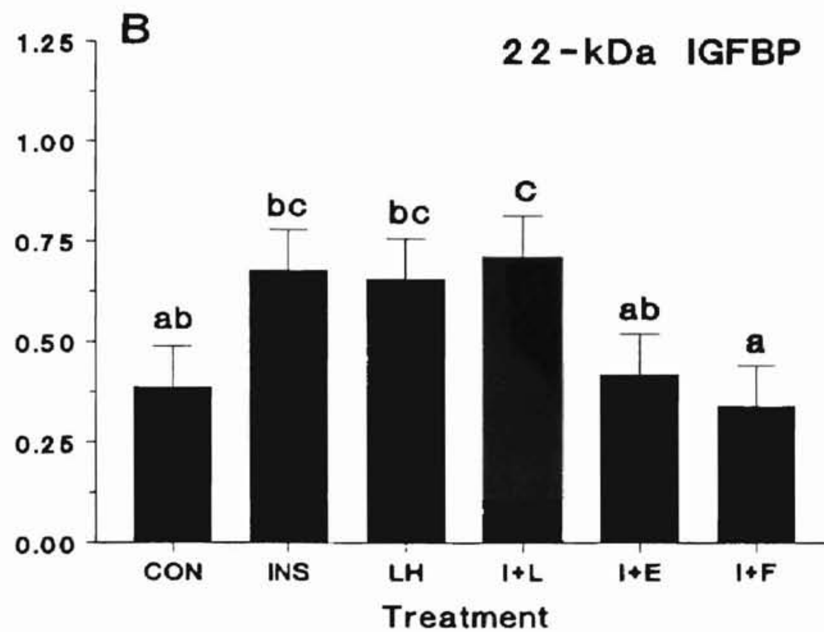


Figure 6. Effect of insulin, FSH, EGF, and bFGF on the 27-34-kDa IGFBP (IGFBP-2/5) production (arbitrary densitometric units/10^5 cells/24 h) by granulosa cells from small follicles (1-5 mm). Granulosa cells were cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin (INS), 50 ng/mL of FSH (FSH), 100 ng/mL of insulin plus 50 ng/mL of FSH (I + FSH), 10 ng/mL of EGF plus 100 ng/mL of insulin (I + E), 10 ng/mL of bFGF plus 100 ng/mL of insulin (I + F) or no additions (CON) for an additional 24 h. Values are means from three separate experiments. Means without a common letter differ ($p < .05$).

Granulosa Cells

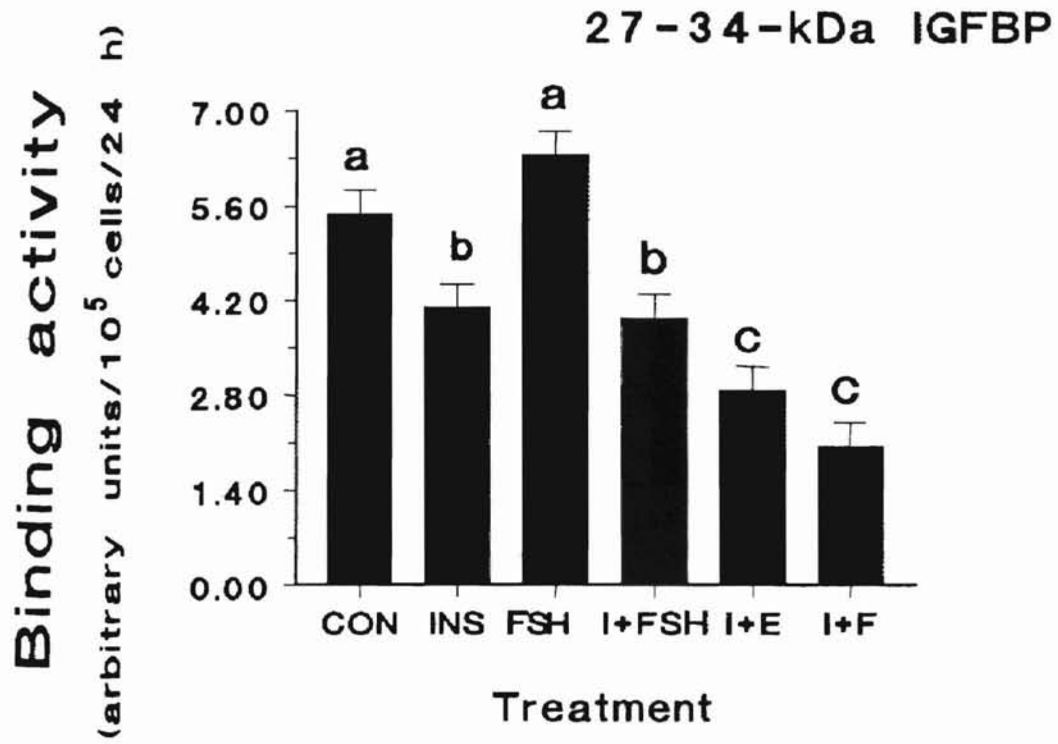


Figure 7. Effect of insulin, glucagon, and cortisol on production of the 27-29-kDa (IGFBP-5, A), 24-kDa (IGFBP-4, B), and 22-kDa (IGFBP-4, C) IGFBP (arbitrary densitometric units/10^5 cells/24 h) by thecal cells from large follicles ($\geq$ 8 mm). Thecal cells were cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin (INS), 30 ng/mL of cortisol (COR), 100 ng/mL of insulin plus 30 ng/mL of cortisol (I + C), 300 pg/mL of glucagon (GLU), 100 ng/mL of insulin plus 300 pg/mL of glucagon (I + G) or no additions (CON) for an additional 24 h. Values are means from three separate experiments. Means without a common letter differ ($p < .05$).

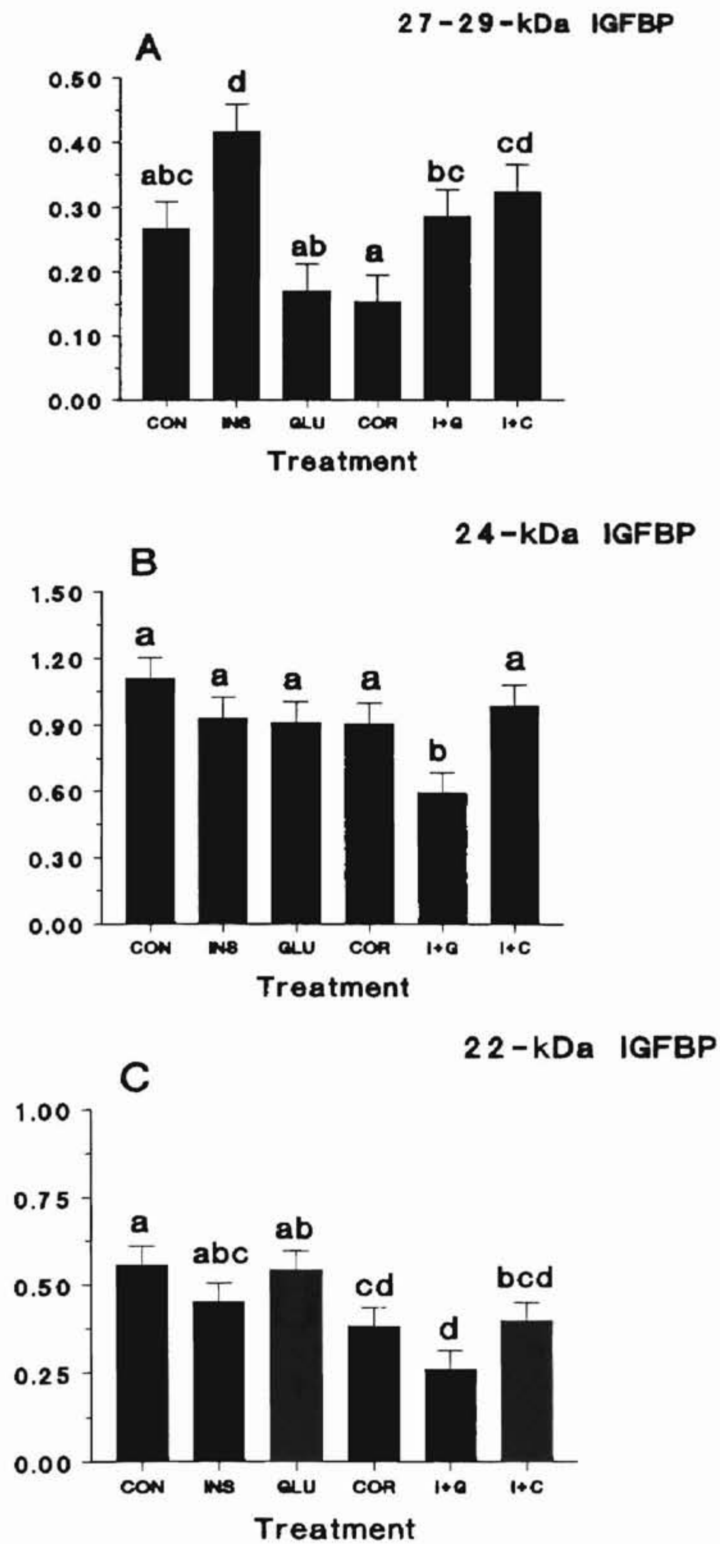

Thecal cells
Binding activity (arbitrary units/10^5 cells/24 h)
27-29-kDa IGFBP
A
0.50
0.40
0.30
0.20
0.10
0.00
abc
d
ab
a
bc
cd
CON
INS
GLU
COR
I+G
I+C
Treatment
24-kDa IGFBP
B
1.50
1.20
0.90
0.60
0.30
0.00
a
a
a
a
b
a
CON
INS
GLU
COR
I+G
I+C
Treatment
22-kDa IGFBP
C
1.00
0.75
0.50
0.25
0.00
a
abc
ab
cd
d
bcd
CON
INS
GLU
COR
I+G
I+C
Treatment

Figure 8. Effect of insulin, glucagon, and cortisol on production of the 27-34-kDa (IGFBP-2/5, A), 20-kDa (IGFBP-4, B), and 18-kDa (IGFBP-4, C) IGFBP (arbitrary densitometric units/10^5 cells/24 h) by granulosa cells from small follicles (1-5 mm). Granulosa cells were cultured for 2 days in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 100 ng/mL of insulin (INS), 30 ng/mL of cortisol (COR), 100 ng/mL of insulin plus 30 ng/mL of cortisol (I + C), 300 pg/mL of glucagon (GLU), 100 ng/mL of insulin plus 300 pg/mL of glucagon (I + G) or no additions (CON) for an additional 24 h. Values are means from three separate experiments. Means without a common letter differ ($p < .05$).

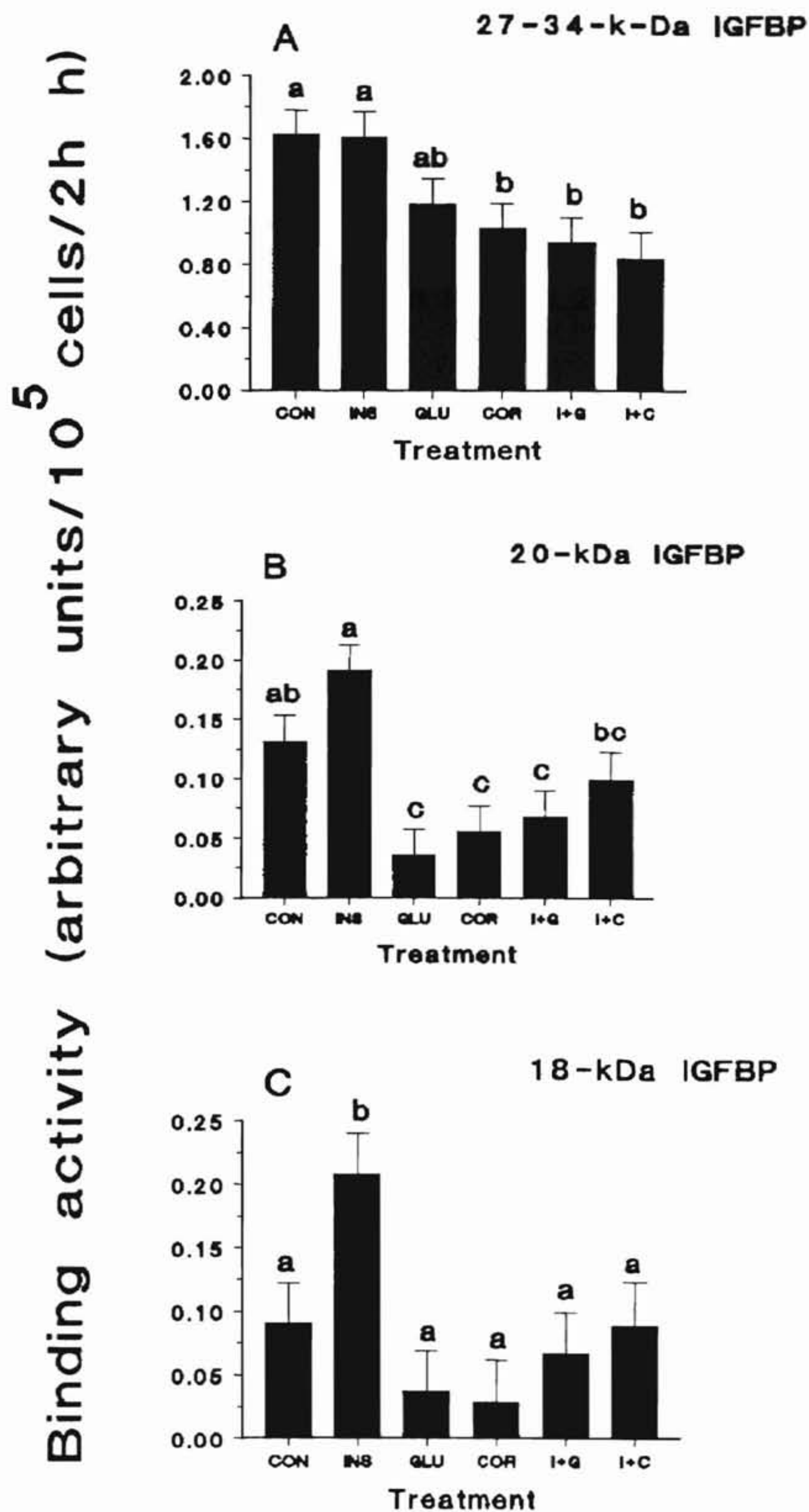

Granulosa Cells
Binding activity (arbitrary units/10^5 cells/2h h)
A
27-34-k-Da IGFBP
2.00
1.60
1.20
0.80
0.40
0.00
a
a
ab
b
b
b
CON
INS
GLU
COR
I+G
I+C
Treatment
B
20-kDa IGFBP
0.25
0.20
0.15
0.10
0.05
0.00
ab
a
c
c
c
bc
CON
INS
GLU
COR
I+G
I+C
Treatment
C
18-kDa IGFBP
0.25
0.20
0.15
0.10
0.05
0.00
a
b
a
a
a
a
CON
INS
GLU
COR
I+G
I+C
Treatment

Table 3

Effect of LH, insulin, EGF, and bFGF on thecal cell proliferation and production of IGFBP-3, -2 and the 24-kDa IGFBP (IGFBP-4; Experiment 2A)

		Arbitrary densitometric units/10^5 cells/24 h		
Treatment	Cell number ($x\ 10^5$/well)[1]	IGFBP-3[2]	IGFBP-2	24-kDa IGFBP[2]
Control	3.211 ± .098[a]	.333 ± .094[a]	.492 ± .082	.505 ± .087[a]
Insulin	4.059 ± .098[b]	.493 ± .094[ab]	.707 ± .082	.754 ± .087[bc]
LH	3.294 ± .098[a]	.496 ± .094[ab]	.508 ± .082	.747 ± .087[abc]
Insulin + LH	4.026 ± .098[b]	.639 ± .094[b]	.772 ± .082	.828 ± .087[c]
Insulin + EGF	6.083 ± .098[c]	.661 ± .094[b]	.605 ± .082	.589 ± .087[abc]
Insulin + bFGF	6.427 ± .098[d]	.713 ± .094[b]	.504 ± .082	.531 ± .087[ab]

Data are mean ± SE of 3 separate experiments.
[1]Means without a common letter differ ($p < .05$).
[2]Means without a common letter differ ($p < .10$).

Table 4

Effect of FSH, insulin, EGF, and bFGF on granulosa cell proliferation and production of the 20-kDa (IGFBP-4) and 18-kDa (IGFBP-4) IGFBP (Experiment 2B)

		Arbitrary densitometric units/10^5 cells/24 h	
Treatment	Cell number (x 10^5/well)[1]	20-kDa IGFBP	18-kDa IGFBP
Control	.448 ± .065[a]	.107 ± .024	.083 ± .024
Insulin	1.435 ± .065[b]	.114 ± .024	.098 ± .024
FSH	.448 ± .065[a]	.143 ± .024	.134 ± .024
Insulin + FSH	1.537 ± .065[b]	.115 ± .024	.102 ± .024
Insulin + EGF	2.018 ± .065[c]	.105 ± .024	.074 ± .024
Insulin + bFGF	2.072 ± .065[c]	.057 ± .024	.052 ± .024

Data are mean ± SE of 3 separate experiments.
[1]Means without a common letter differ ($p < .05$).

Table 5

Effect of insulin, glucagon, and cortisol on thecal cell proliferation and production of IGFBP-3 and –2 (Experiment 3A)

		Arbitrary densitometric units/10^5cells/24 h	
Treatment	Cell number ($x\ 10^5$/well)[1]	IGFBP-3	IGFBP-2[2]
Control	1.796 ± .089[a]	.186 ± .036	.137 ± .024[ab]
Insulin	2.613 ± .089[c]	.173 ± .036	.186 ± .024[b]
Glucagon	1.993 ± .089[ab]	.117 ± .036	.100 ± .024[a]
Cortisol	2.116 ± .089[ab]	.105 ± .036	.094 ± .024[a]
Insulin + Glucagon	2.272 ± .089[b]	.121 ± .036	.147 ± .024[ab]
Insulin + Cortisol	2.870 ± .089[c]	.229 ± .036	.137 ± .024[ab]

Data are mean ± SE of 3 separate experiments.
[1]Means without a common letter differ ($p < .05$).
[2]Means without a common letter differ ($p < .10$).

Table 6

Summary of the effects of hormone treatments on IGFBP production by bovine thecal cells[a,b]

Hormone	IGFBP-3 (42-44-kDa)	IGFBP-2 (34-kDa)	IGFBP-5 (27-29-kDa)[c]	IGFBP-4 (24-kDa)	IGFBP-4 (22-kDa)
Insulin[a]	No effect	No effect	Increase[a]	No effect	No effect
LH[ab]	No effect	No effect	No effect	No effect	No effect
EGF[b]	No effect	No effect	No effect	No effect	No effect
bFGF[b]	No effect	No effect	Decrease	No effect	Decrease
Glucagon[ab]	No effect	No effect	No effect[a]/ Decrease[b]	No effect[a]/ Decrease[b]	Decrease[b]/ No effect[a]
Cortisol[ab]	No effect	No effect	No effect	No effect	No effect[b]/ Decrease[a]

[a]Effects are compared with control values (basal).
[b]Effects are compared with insulin-treated values.
[c]The major IGFBP produced by thecal cells.

Table 7

Summary of the effects of hormone treatments on IGFBP production by bovine granulosa cells[ab]

Hormone	IGFBP-2/5	IGFBP-4	
	(27-34-kDa)[c]	20-kDa	18-kDa
Insulin[a]	Decrease/No effect	No effect	Increase
FSH[ab]	No effect[a]/Decrease[b]	No effect	No effect
EGF[b]	Decrease	No effect	No effect
bFGF[b]	Decrease	No effect	No effect
Glucagon[ab]	No effect[a]/Decrease[b]	Decrease	No effect[a]/Decrease[b]
Cortisol[ab]	Decrease	Decrease	No effect[a]/Decrease[b]

[a]Effects are compared with control values (basal).

[b]Effects are compared with insulin-treated values.

[c]The major IGFBP produced by granulosa cells.

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CHAPTER IV

Summary and Conclusions

Folliculogenesis is a complex and dynamic process. A multitude of factors interact to ultimately result in one selected follicle to ovulate while the remaining cohort of follicles will regress and die. During normal cyclicity, IGF-I enhances steroidogenesis of the granulosa and thecal cells although ovarian IGF-I levels remain constant. However, serum IGF-I concentrations decreases in non-cyclic cows, suggesting that IGF-I is important for sustained reproduction. Indeed, IGF-I as well as its IGFBPs do influence steroidogenesis of the follicle. The ultimate question however remains to be determined. That is, what are the factors that determine selection of the follicle?

The present study evaluated the effects of hormones applied to cultured thecal or granulosa cells to determine the hormonal regulation of IGFBP production. IGFBP-2, -4, and -5 were produced by the bovine thecal and granulosa cells. IGFBP-3 was also produced by the thecal cells, but not the granulosa cells. Moreover, glucagon, cortisol, insulin, bFGF, and EGF all appeared to impact IGFBP production but each IGFBP was influenced differentially by a different subset of hormones. However, the actual hormone, or complex of hormones which controls IGFBP synthesis was not resolved. The conclusion to obtain from this work is that IGFBPs are indeed influenced by hormones with no one IGFBP influenced synchronously with another. Through a

network of hormones, and possibly proteases, IGFBPs levels are modulated through the estrous cycle, thus determining the bioavailability of IGF-I and ultimately, follicle growth. Thus, by understanding the factors that determine follicle selection, and ultimately ovulation, the loss of productivity from non-cyclic cattle will subside.

VITA

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Thesis: PRODUCTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) BY BOVINE GRANULOSA AND THECAL CELLS: REGULATION BY METABOLIC HORMONES

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