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Insulin-Like Growth Factor Binding Proteins in the Bovine Anterior Pituitary*

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Insulin-like growth factor binding proteins (IGFBPs) were characterized in bovine anterior pituitary tissue, pituitary conditioned media, and serum collected during the preovulatory and early luteal phases of the estrous cycle. Effects of in vitro treatments of pituitaries with luteinizing hormone–releasing hormone (LHRH), estradiol, and progesterone on IGFBP secretion were also evaluated. Predominant IGFBPs detected in anterior pituitary tissue by immunoprecipitation, ligand blotting, and Northern blotting were IGFBP-5 (29 kDa), IGFBP-2 (32 kDa), and IGFBP-3 (36 and 39 kDa doublet). Conditioned culture media contained IGFBP-5, a slightly larger form of IGFBP-2 (33 kDa), the 36- and 39-kDa forms of IGFBP-3, and a more extensively glycosylated form of IGFBP-3 (44 kDa). In serum, IGFBP-5 was not readily detected, and IGFBP-3 (40- and 44-kDa doublet) and IGFBP-2 (34 kDa) were larger than in pituitary tissue. Levels of IGFBP-2, -3, and -5 in pituitary tissue decreased during the preovulatory period and were lowest in the early luteal phase. Treatment with LHRH increased IGFBP-2 levels in media twofold. Estradiol or progesterone did not alter IGFBP secretion in vitro. Predominant IGFBPs produced and released by anterior pituitary tissue were IGFBP-2, -3 and –5. The activity of IGFBPs fluctuates in the pituitary in association with changes in stage of estrous cycle, implicating IGFBPs as potential regulators of gonadotrope function.

Key Words: Bovine; pituitary; insulin-like growth factor binding proteins; luteinizing hormone–releasing hormone.

Introduction

Previous research implicates the insulin-like growth factor (IGF) system as having a role in mediating gonadotropin secretion. IGF-1 alters in vitro secretion of gonadotropins from rat pituitary cells *(1–3)* and several human pituitary adenomas *(4)*. However, the bioavailability of IGF-1 to specific receptors in vivo is subjected to regulation by IGF-binding proteins (IGFBPs) *(5,6)*. Several IGFBPs, as well as IGF-I, have been detected in anterior pituitary tissue from different species *(7–14)*. Potential importance of the IGF system in regulating pituitary function is provided by observations that levels of IGF-1 and IGFBPs in anterior pituitary gland tissue were greater than in the hypophyseal stalk-median eminence, medial basal hypothalamus, preoptic area of the hypothalamus, and liver *(11,12)*. While concentrations of IGF-1 remained relatively constant in anterior pituitary glands collected at different stages of the estrous cycle, levels of IGFBPs were correlated with circulating concentrations of progesterone *(13)*, and expression of IGFBP-2 in anterior pituitary tissue was increased by estradiol treatment in rats *(15)* and ewes *(14)*. Therefore, IGFBP activity, but not IGF-1, appears to fluctuate in the anterior pituitary gland. These fluctuations appear to be associated with ovarian, and possibly hypothalamic, factors that influence anterior pituitary gland function. In addition, IGFBPs observed in pituitary tissue differed from those observed in serum *(13)*, providing evidence that synthesis and/or processing of IGFBPs occurs in the pituitary. However, a thorough characterization of IGFBPs synthesized and released by bovine anterior pituitary tissue during specific stages of the estrous cycle is lacking.

The objectives of the present study were to obtain further knowledge concerning the composition of IGFBPs in the bovine anterior pituitary gland and the potential for their secretion from this tissue during the preovulatory and early luteal phase of the estrous cycle. In addition, the effects of in vitro treatments with luteinizing hormone-releasing hormone (LHRH), estradiol, and progesterone on anterior pituitary release of IGFBPs were evaluated to provide insight into potential mechanisms regulating IGFBP secretion.
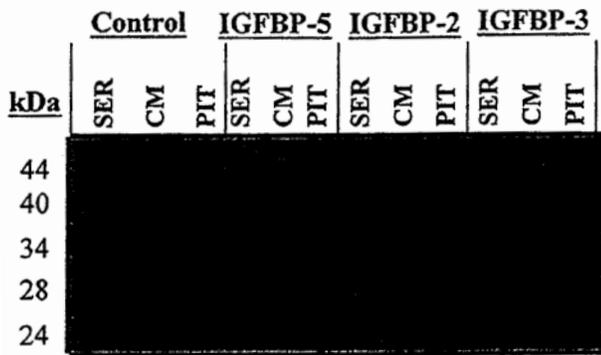
Fig. 1. Ligand blot of serum (SER), pituitary-conditioned medium (CM), and pituitary homogenates (PIT). Samples were subjected to no prior treatment (control) or immunoprecipitation with antibodies against specific IGFBP (as indicated in top line) prior to loading onto the gel. Control samples were loaded onto the gel at 1 μL, 2 mL, and 100 μg total protein equivalent for serum, conditioned medium, and pituitary homogenates, respectively. Quantities of samples subjected to immunoprecipitation were 5 μL, 2 mL, and 200 μg total protein equivalent for serum, conditioned medium, and pituitary homogenates, respectively. The resulting immunoprecipitates of each sample were then loaded onto the gel. The anti-IGFBP-3 antibody used for this procedure exhibits crossreactivity with IGFBP-2.

Results

IGFBPs in Serum, Pituitary Homogenates, and Conditioned Media from Pituitary Cultures

Representative profiles of IGFBPs detected in serum, pituitary homogenates, and conditioned media from cultured pituitary slices are shown in the first three lanes of Fig. 1. The remaining lanes of Fig. 1 depict IGFBP bands immunoprecipitated from samples treated with antibodies to IGFBP-5, -2, and -3. Figure 2 provides another example of IGFBPs detected in anterior pituitary tissue (first lane) and serum (third lane) and depicts the influence that treatment with N-Glycosidase F has on the mass of these binding proteins.

In pituitary tissue and conditioned medium, IGFBP-5 was detected by immunoprecipitation as an ~29-kDa protein. This protein was smaller ($p < 0.01$) than a faint 30- to 31-kDa band evident in nonimmunoprecipitated serum samples and was not affected by N-Glycosidase F (cf. lanes 1, 2, and 3 in Fig. 2). The 30- to 31-kDa IGFBP in serum was not precipitated with any antibodies used.

All three sample types contained an IGFBP that was precipitated by antibodies against IGFBP-2. However, the size of IGFBP-2 differed ($p < 0.05$) among pituitary tissue (~32 kDa), conditioned medium (~33 kDa), and serum (34 kDa). A 29- to 30-kDa band was also faintly visible in pituitary homogenates and conditioned medium immunoprecipitated with the IGFBP-2 antibody, but not in serum.

Because the IGFBP-3 antiserum crossreacted with IGFBP-2, proteins precipitated by antibodies to IGFBP-2 were also evident in samples precipitated with the IGFBP-3 antiserum. In addition, the IGFBP-3 antiserum precipitated proteins of approx 44, 39–40, and 36 kDa. As summarized in
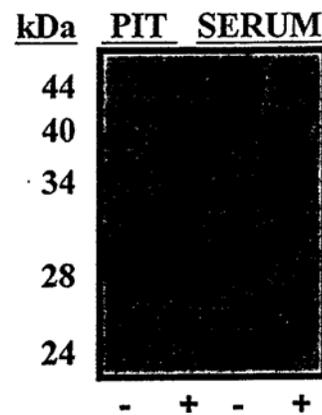

Fig. 2. Ligand blot of pituitary homogenates (PIT) and serum samples that were untreated (−) or treated with N-Glycosidase F (+) to remove N-linked glycosylations prior to loading on the gel. The relative mass of IGFBP-3 (top two bands in each untreated sample) in both samples and the 28-kDa IGFBP-4 in serum (second band from bottom of untreated sample) were reduced after deglycosylation.

Fig. 3, the 44-kDa form of IGFBP-3 was not detected in ligand blots of pituitary homogenates not subjected to immunoprecipitation (see third lane of Fig. 1, first lane of Fig. 2) but was detected in all serum samples. The size of the 39- to 40-kDa IGFBP-3 detected in homogenates and conditioned medium from pituitaries was smaller ($p < 0.01$) than that observed in serum. The 36-kDa form of IGFBP-3 was detected in pituitary samples, but not serum. Treatment with N-Glycosidase F reduced the mass of IGFBP-3 in pituitary tissue and serum (Fig. 2).

Two additional bands were observed in ligand blots that were not detected after immunoprecipitation. These bands were consistent in size with glycosylated (28 kDa) and nonglycosylated (24 kDa) forms of IGFBP-4 identified in follicular fluid and serum of cattle *(18,27)*. In serum, the 28-kDa band was reduced in size by deglycosylation (Fig. 2). The 28- and 24-kDa bands were detected in all serum samples. By contrast, a 27-kDa band ($p < 0.01$ for size compared with the 28-kDa in serum) was detected only in 55% of tissue homogenates and 61% of conditioned medium samples (Fig. 3). A 24-kDa protein was detected in 33% of conditioned medium samples but was not detected in tissue from which conditioned medium was generated.

The proportion of total binding activity accounted for by each IGFBP differed ($p < 0.05$) among sample types (Fig. 3). In serum, IGFBP-3 accounted for the majority (58%) of total IGF binding activity. By contrast, IGFBP-5 accounted for the largest proportion of IGF binding activity in conditioned medium (46%) and tissue homogenates (57%).

Effects of LHRH, Estradiol, and Progesterone on In Vitro Secretion of IGFBPs, LH, and Follicle-Stimulating Hormone

Incubation of pituitary slices with LHRH resulted in a twofold increase ($p < 0.05$) in IGFBP-2 binding activity in
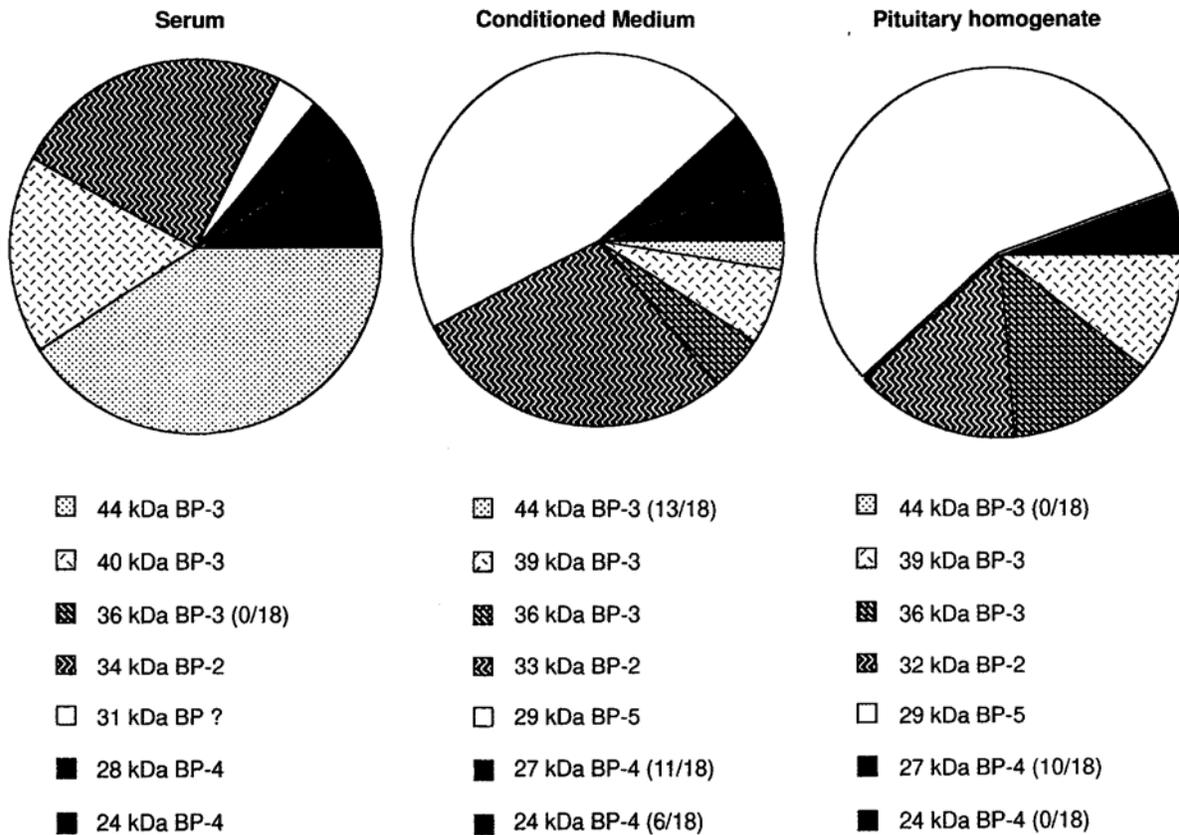
Serum   **Conditioned Medium**   **Pituitary homogenate**

⊞ 44 kDa BP-3	⊞ 44 kDa BP-3 (13/18)	⊞ 44 kDa BP-3 (0/18)
◺ 40 kDa BP-3	◺ 39 kDa BP-3	◺ 39 kDa BP-3
▨ 36 kDa BP-3 (0/18)	▨ 36 kDa BP-3	▨ 36 kDa BP-3
▩ 34 kDa BP-2	▩ 33 kDa BP-2	▩ 32 kDa BP-2
☐ 31 kDa BP ?	☐ 29 kDa BP-5	☐ 29 kDa BP-5
■ 28 kDa BP-4	■ 27 kDa BP-4 (11/18)	■ 27 kDa BP-4 (10/18)
■ 24 kDa BP-4	■ 24 kDa BP-4 (6/18)	■ 24 kDa BP-4 (0/18)

Fig. 3. Relative percentage of the total IGF-1 binding activity accounted for by individual IGFBPs detected in serum, conditioned medium, and pituitary slice homogenate samples from 18 animals. Numbers in parentheses represent the proportion of samples exhibiting binding in that area; no number denotes that samples from all 18 animals exhibited binding. Identification of proteins is based on immunoblot analysis (Fig. 1). A question mark indicates that identity of the 29- to 31-kDa protein in serum was not established. The percentage of total binding activity accounted for by the individual IGFBPs varied ($p < 0.05$) among the three types of samples.

conditioned medium (5.0 ± 0.5 vs 2.7 ± 0.4 arbitrary units). The IGFBP-2 binding activity in tissue treated with LHRH (6.4 ± 0.4) was only slightly greater ($p < 0.1$) than tissue cultured without hormone treatment (5.5 ± 0.4). Treatment with LHRH did not influence binding activity of other IGFBPs in conditioned medium or tissue. Incubation of pituitary tissue with estradiol or progesterone did not influence IGFBPs in conditioned medium and pituitary homogenates.

Treatment of pituitary tissue with LHRH resulted in greater ($p < 0.01$) concentrations of LH (1494 ± 74 ng/mL) and follicle-stimulating hormone (FSH) (91 ± 5 ng/mL) released into the conditioned medium than in nontreated cultures (1071 ± 76 ng of LH/mL; 61 ± 5 ng of FSH/mL). Release of gonadotropins into culture medium was not influenced ($p > 0.3$) by either estradiol or progesterone. Concentrations of FSH (pooled mean $\pm$ SEM = 32 ± 11 ng/mg) and LH (680 ± 280 ng/mg) in pituitary slices were not different ($p > 0.15$) among treatment groups.

Effects of Time After Prostaglandin $F_{2\alpha}$ on IGFBPs

The IGF binding activity in pituitary tissue collected from animals slaughtered at different times after prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) is summarized in Fig. 4. Binding activity by each

IGFBP decreased as time after PGF$_{2\alpha}$ increased. The IGFBP-3 ($r = 0.50$) and IGFBP-5 ($r = 0.56$) binding activities in pituitary tissue were associated ($p < 0.05$) with circulating concentrations of progesterone during the preovulatory period (i.e., d 1 and 2 after PGF$_{2\alpha}$), but this association was not evident ($p > 0.1$) when data from all three time periods were evaluated. Pituitary IGFBP-2 tended ($p < 0.08$) to be inversely ($r = -0.45$) associated with estradiol concentrations of follicular fluid during the preovulatory period.

Northern Blot Detection of IGFBP-5 mRNA

To provide additional evidence for pituitary synthesis of IGFBP-2, -3, and -5, total cellular RNA from representative pituitary samples from different animals ($n \geq 5$) was subjected to Northern blot analysis (Fig. 5). A 1.4-kb transcript was detected for IGFBP-2 and a 2.4-kb transcript for IGFBP-3. Transcripts of 6 and 1.8 kb were detected for IGFBP-5 mRNA.

Discussion

The present study provides evidence that IGFBP-2, -3, and -5 are synthesized and secreted by bovine anterior pitu-
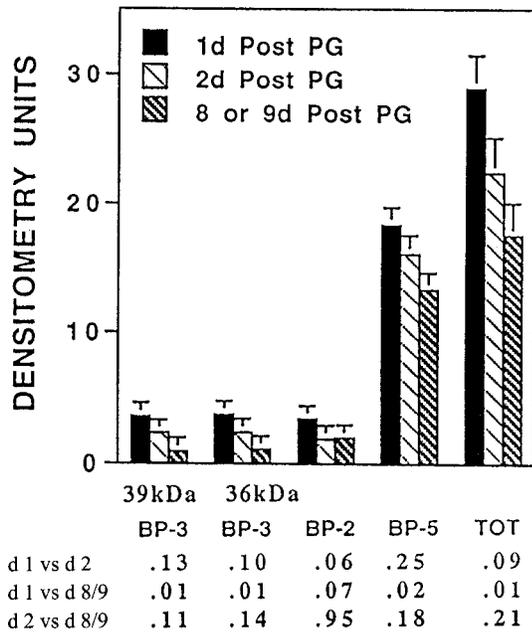
	39kDa	36kDa			
	BP-3	BP-3	BP-2	BP-5	TOT
d 1 vs d 2	.13	.10	.06	.25	.09
d 1 vs d 8/9	.01	.01	.07	.02	.01
d 2 vs d 8/9	.11	.14	.95	.18	.21

Fig. 4. Activity of IGFBP in pituitary tissue from eight cows slaughtered at either 1, 2, or 8 to 9 d after $PGF_{2\alpha}$ (PG) to provide pituitary tissue from preovulatory (1 or 2 d after $PGF_{2\alpha}$) or early luteal (8 or 9 d after $PGF_{2\alpha}$) phases of the estrous cycle. The *p* values for each pairwise comparison of mean IGF-binding activity are given below the graph.

Fig. 5. Representative lanes from Northern blots of 15 µg total RNA isolated from anterior pituitaries. Blots were probed with cRNAs for IGFBP-2, -3, and -5 as indicated above each lane. Location of the 18S ribosomal RNA is indicated on the left. A single transcript of ~1.4 kb was detected for IGFBP-2. A transcript of ~2.4 kb was detected for IGFBP-3. Transcripts for IGFBP-5 were detected at approx 6 kb and in close proximity to the 18S ribosomal RNA (~1.8 kb).

itary tissue. Levels of IGFBP activity in the anterior pituitary gland declined from the preovulatory through the early postovulatory period, and LHRH stimulated pituitary release of IGFBP-2 in vitro. These results provide additional basis for the hypothesis that IGFBPs may have an important role in controlling reproductive function by mediating IGF-1 influences on gonadotrope function.

The IGFBPs observed in pituitary tissue differed from those in serum in apparent molecular weight (IGFBP-2 and -3) and relative abundance. During the physiologic states evaluated, IGFBP-5 accounted for the majority of total IGF-1 binding activity in bovine anterior pituitary tissue. The remainder of the binding activity was primarily owing to IGFBP-3 and -2, with some samples exhibiting binding by a protein similar in size to IGFBP-4. In contrast to pituitary samples, very little IGFBP-5 was detected in serum as reported previously in cattle (18) and other species (5); IGFBP-3 accounted for the majority of total binding activity detected in serum of cattle with binding by IGFBP-2 and -4 ranking second and third, respectively; the apparent sizes of IGFBP-2 and -3 were larger in serum than in anterior pituitary tissue; and serum contained a 30- to 31-kDa protein that was not detected in pituitary samples. Previous research indicates that this 30- to 31-kDa protein may be IGFBP-1 (19), but an antibody to characterize this protein in cattle was not available.

The proportionately large amount of IGFBP-5 activity and smaller forms of IGFBP-2 and -3 detected in pituitary
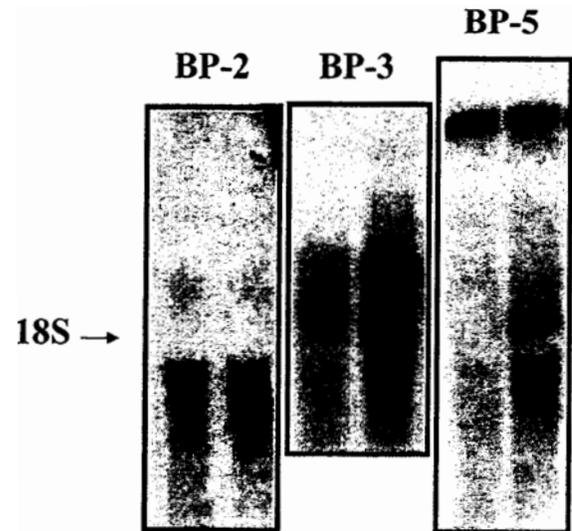
tissue, as compared to serum, are consistent with intrapituitary synthesis and processing of IGFBPs. Detection of transcripts for IGFBP-2, -3, and -5 provides additional evidence that synthesis of these IGFBPs occurs in pituitary tissue. Transcripts for IGFBP-2, -3, -4, and -5, but not IGFBP-1, were detected in rat anterior pituitary tissue (9) and IGFBP-2 mRNA, and protein was detected in ovine pituitary tissue (14). Rodent pituitaries secrete IGFBPs (7,8,10); however, the cited studies did not determine whether tissue and secreted forms of IGFBPs differed in size, as was evident for IGFBP-2 and -3 in the present study. Research in sheep demonstrated that forms of IGFBP-2 and -3 in pituitary and stalk medium eminence tissue were smaller than those in serum (12,14,20), but these studies did not evaluate secretion of IGFBPs. Three potential N-linked glycosylation sites exist on IGFBP-3 (5), and the present study demonstrated that pituitary conditioned medium and serum have an increased predominance of more extensively glycosylated forms of IGFBP-3 than pituitary tissue. Reasons for differences in relative size of IGFBP-2 in pituitary tissue, conditioned medium, and serum are not evident but may not be owing to glycosylation because bovine IGFBP-2 was not predicted to contain O- or N-linked glycosylation sites (21). Therefore, other posttranslational modifications of IGFBP-2 may account for differences among these three sample types. Nevertheless, these studies provide evidence that IGFBP-2, -3, and -5 are synthesized and processed in bovine pituitary tissue.

The fact that pituitary tissue contains smaller forms of IGFBP-2 and -3 than serum emphasizes the need to characterize these proteins by means other than size alone. Interestingly, the size of these proteins observed in bovine uterine tissue *(19)* and ovarian follicular cells *(16)* was not different from serum. Therefore, tissue-specific differences in synthesis, storage, and processing may exist.

Mechanisms regulating the synthesis and secretion of IGFBPs in the anterior pituitary gland remain to be established. Short-term treatment of cultured pituitary tissue with LHRH increased secretion of IGFBP-2 as well as FSH and LH. To our knowledge, this is the first report demonstrating that LHRH stimulates pituitary secretion of IGFBPs, and a role of IGFBPs in mediating hypothalamic control of pituitary function is suggested. Whether this effect will be evident *in situ* remains to be established. In contrast to LHRH, short-term treatment of cultured pituitary tissue with estradiol or progesterone did not influence secretion of IGFBPs or gonadotropins. Because of the short duration of treatments, conclusions regarding the effects, or lack thereof, of these hormones are limited to influences on secretion. Longer-term (≥ 2 wk) in vivo treatment of ovariectomized animals with estradiol increased pituitary binding activity and mRNA for IGFBP-2 in rats *(15)* and ewes *(14)*. In prepubertal cattle, ovariectomy resulted in a significant decrease in pituitary levels of IGFBP-2, -3, and -5 (unpublished observation). However, treatment of ovariectomized animals with estradiol prevented decreases in levels of IGFBP-2 and IGFBP-5, but not IGFBP-3. Estradiol treatment of ovariectomized ewes also increased IGFBP-2, -3, and -5 in the stalk median eminence *(14)*, another example of steroid regulation of IGFBPs in the hypothalamic-pituitary axis.

A limitation of in vitro experiments and studies utilizing ovariectomized animals is that these experimental approaches do not account for the total endocrine milieu and the complex interactions among different hormones that exist in an intact animal. Observations that IGFBP activity decreased from d 1 to d 2 after $PGF_{2\alpha}$ (i.e., preovulatory period) and were even lower 8 to 9 d after $PGF_{2\alpha}$ (i.e., early luteal phase) provide insight that the interaction among ovarian and hypothalamic hormones may by important in regulating IGFBP activity in the anterior pituitary gland. During the preovulatory period, increased estradiol and LHRH stimulation of the pituitary would be expected. However, IGFBP in the tissue decreased during this period. Thus, factors other than estradiol and LHRH are also likely to regulate IGFBP activity. Levels of IGFBP-3 and -5 in the pituitary were associated with decreases in circulating concentrations of progesterone whereas IGFBP-2 levels tended to be inversely associated with estradiol concentration in the ovulatory follicle. Positive correlations among IGFBP-2, -3, and -5 in the pituitary and circulating concentrations of progesterone during the estrous cycle were observed previously *(13)*. Based on observations of IGFBPs in the intact animals and results from the in vitro experiment and stud-

ies utilizing ovariectomized animals, it appears that IGFBP activity in anterior pituitary tissue is regulated by interactions among endocrine input from the hypothalamus and gonad and is dependent on the balance of effects on synthesis and secretion.

While the physiologic significance of these observations remains to be confirmed, actions that IGFBPs have in altering numerous other biologic processes have been studied in a wide variety of experimental models (see refs. *5* and *6* for review). Depending on the experimental system and conditions used, IGFBPs can either inhibit or potentiate the actions of IGFs. Inhibition of these actions is attributed to the high affinity that IGFBPs bind IGFs, thereby preventing IGFs from interacting with IGF receptors. Potentiation of IGF actions involves interaction of individual binding proteins (i.e., IGFBP-1, -3, and -5) with the cell surface, thereby increasing the bioavailability of IGFs in the microenvironment surrounding cells and enhancing ligand-receptor association. In some cases, the ability of IGFBP to potentiate IGF actions is influenced by the phosphorylated state of the binding protein (IGFBP-1) or proteolytic cleavage of the IGFBP, which decreases affinity for IGFs. Because binding affinities for IGF-1 and IGF-2 differ among individual IGFBPs, the composition of IGFBPs or the posttranslational processing of IGFBPs in anterior pituitary tissue may markedly influence the interaction of IGFs with IGF receptors on cells in the anterior pituitary. Early research demonstrated that titration curves of IGF binding in conditioned medium from rat pituitary tissue were not always parallel to those observed for serum *(7)*, indicating that the equilibrium among IGFs and IGFBPs in the anterior pituitary differed from that existing in the circulation. Therefore, the amounts and types of IGFBPs synthesized and processed within a given tissue or cell type regulates tissue responsiveness to the IGFs. In addition to modulating the bioavailability of IGFs, IGFBP-1, -2, -3, and -5 may also alter cellular function in an IGF-independent fashion through interaction with intergrin receptors (IGFBP-1) or other cell surface binding sites (IGFBP-3 and -5) (*see* refs. *5* and *6* for reviews).

It is well established that LHRH and gonadal steroids interact to regulate synthesis and secretion of gonadotropins. Likewise, LHRH and ovarian steroids also appear to interact in regulating pituitary levels of IGFBPs. Present and previous research *(13)* indicate an inverse relationship among activities of individual IGFBPs in the pituitary and circulating concentrations of LH throughout the estrous cycle. Because IGF-1 can enhance LHRH-stimulated secretion of gonadotropin from pituitary cells *(1–4)*, the inverse associations of pituitary IGFBPs with circulating concentrations of LH are consistent with an inhibitory role of IGFBPs on IGF-1-stimulated gonadotropin secretion. These observations provide a basis for the hypothesis that IGFBPs act within the anterior pituitary to mediate endocrine feedback loops controlling gonadotrope function. Steroid inhibition

of LH secretion may be mediated, at least in part, by increases in levels of IGFBP-2, -3, and -5 in the pituitary, which, in turn, suppress IGF-1 augmentation of LH secretion. The effects that estradiol and progesterone have on IGFBPs may be direct or indirect as documented for steroid effects on pituitary gonadotrope function. While the implications of LHRH-stimulated release of IGFBP-2 remain to be determined, increases in IGFBP-2 release may be involved in the sequestering of IGF-1 and thereby the culmination of each LH pulse in response to LHRH.

Collectively, the present and discussed studies support the hypothesis that IGFBPs act within the pituitary to modulate hypothalamic and ovarian endocrine inputs regulating reproductive function. However, additional research will be required to substantiate this hypothetical model.

Materials and Methods

Collection and Processing of Pituitary Tissue

Mature cycling beef cows with a corpus luteum present on their ovaries were injected with ($PGF_{2\alpha}$) (Lutalyse; Upjohn, Kalamazoo, MI) to induce luteolysis. Cows were then slaughtered at 1 ($n = 8$), 2 ($n = 8$), or 8 to 9 ($n = 8$) d after injection to provide animals in the preovulatory (d 1 and 2) and early luteal (d 8 and 9) stages of the estrous cycle. These time points were selected to characterize more precisely the decline in IGFBP activity previously (13) reported for cows classified as preovulatory (d 17–21) or postovulatory (d 1–5).

Blood samples were collected from each animal immediately after slaughter, and stored overnight at 4°C, and serum was harvested and stored at −20°C for analysis. Anterior pituitary glands and ovaries were collected within 20–30 min after exsanguination at slaughter. Trimmed anterior pituitary glands were either prepared for culture or snap-frozen in liquid nitrogen. Ovaries were collected for determination of follicular fluid concentrations of estradiol in large follicles (all follicles >8 mm diameter) and corpus luteum weights to verify stage of estrous cycle at slaughter.

Anterior pituitary glands from 15 cows ($n = 5/PGF_{2\alpha}$ group) were subjected to in vitro culture. Four midsagittal slices, 1 to 2 mm thick, from each gland were placed in sterile Petri dishes containing 3 mL of a 1:1 mixture of Dulbecco's modified Eagle's medium:Ham's F12 (DMEM/F12) (Gibco-BRL, Gaithersburg, MD). Medium was changed four times at 45-min intervals to wash the slices. Pituitary slices were then placed into six-well culture plates containing 3 mL of DMEM/F12 per well. Tissue slices were incubated for three 40-min intervals under 95% air, 5% CO_2 at 37°C. Culture medium was collected after each 40-min period and replaced with new medium. Medium from the first two 40-min culture periods was used to determine baseline levels of IGFBP production. Medium used for the last 40-min period contained either no hormone (control),

LHRH (66 ng/mL) (Sigma, St. Louis, MO), estradiol (E_2) 60 pg/mL), or progesterone (P_4) (10 ng/mL). These hormone concentrations were previously shown to influence LH secretion in cultured bovine pituitary cells (22). Treatments during the last 40 min were randomly applied to one of the four slices from each animal. After culture, conditioned media were centrifuged and frozen. Pituitary slices were blotted dry, weighed, snap-frozen in liquid nitrogen, and stored at -70°C for subsequent analysis of IGFBPs and concentrations of LH and FSH.

Analysis of Hormones and IGFBPs

Concentrations of LH (23) and FSH (24) in medium and homogenates of cultured pituitary tissue were determined by radioimmunoassay. All samples were analyzed in one assay for each hormone with a coefficient of variation (CV) of 6 and 8% for LH and FSH, respectively. Sensitivities (>5% displacement) of each assay were 0.2 ng/tube. Concentrations of estradiol in follicular fluid samples and progesterone in serum were determined as described previously (16), except that progesterone was extracted from serum with heptane (17) prior to being assayed. All samples were analyzed in one assay for estradiol or progesterone (CV = 8 and 10%, respectively) with a sensitivity (>5% displacement) of 0.2 and 5 pg/tube, respectively.

Ligand blot procedures (25) were used as described (19) to evaluate IGFBPs in serum, conditioned medium from pituitary cultures, and tissue homogenates subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (26). Serum samples and a pool of bovine follicular fluid (used as a positive control on each gel) were analyzed at a volume of 1 μL of sample diluted in 49 μL of gel-loading buffer (62.5 m*M* Tris base; 2% SDS; 0.02% bromophenol blue; 10% glycerol; pH 6.8). IGFBPs in 1 mL of conditioned medium from each incubation were concentrated by precipitation with 2 vol of ethanol and centrifugation at 12,000g for 15 min. Resulting pellets were resuspended in 50 μL of gel-loading buffer and loaded onto the gels. Pituitary tissue was homogenized at 100 mg of tissue/ mL of buffer (0.1% SDS, 1% cholic acid, 0.1 m*M* phenylmethylsulfonyl fluoride). Total protein concentration in pituitary homogenates was determined by the BCA procedure (Pierce, Rockford, IL), and samples were diluted with gel-loading buffer to equivalent quantities of total protein (100 μg) in a total volume of 50 μL. All samples were boiled 4 min and placed on ice for 5–10 min prior to loading into wells of a 4% stacking gel layered over a 12% separating gel. Proteins were electrophoretically transferred from the gel onto nitrocellulose membranes. IGFBPs were detected by probing with IGF-1 (DRG010; Bachem, Torrance, CA) radiolabeled with ^{125}I. Preliminary studies indicated that probing membranes with a mixture of radiolabeled IGF-1 and IGF-2 did not reveal any additional IGFBPs, as those detected with IGF-1 alone. Autoradiographic film was ex-

posed to membranes. Band intensity and relative migration distance of individual binding proteins were determined by scanning laser densitometry. For the in vitro experiment, 17 samples were generated for each cow: 1 serum, 12 conditioned medium (3 collections for each of four treatments), and 4 pituitary homogenates (1 for each treatment). The gel and transfer apparatuses used were capable of running a total of 48 samples in one set of gels ($n \leq 4$) at one time. Seven sets of gels were run to allow comparisons of in vitro treatments on IGFBP in media and pituitary homogenates. Serum samples were also included in this group of gels to allow comparison of IGFBPs among the different types of samples. All samples from one animal were included within a set of gels so that the aforementioned comparisons could be made on a within-cow basis. An additional set of gels was run on pituitary homogenates to allow analysis of the effects of time after $PGF_{2\alpha}$ on IGFBPs.

Identification of IGFBPs in serum (5 µL), anterior pituitary homogenates (200 µg of protein), and conditioned medium (2 mL precipitated with 2 vol of ethanol) was determined by immunoprecipitation and subsequent ligand blot analysis *(18,27)*. Samples were immunoprecipitated using a partially purified (1 mg of IgG/mL) rabbit antirat IGFBP-2 antibody *(28)*, rabbit antibovine IGFBP-3 antiserum (RK53, provided by Dr. D. Clemmons, University of North Carolina, Chapel Hill), or rabbit antihuman IGFBP-5 antiserum (Upstate Biotechnology, Lake Placid, NY). Samples were incubated overnight at 4°C with 2 µL of antibody solution or antiserum after which preprecipitated goat antirabbit IgG antibody suspension was added *(27)*. Samples were subsequently centrifuged at 12,000*g* for 15 min. Precipitates were resuspended in gel-loading buffer and subjected to SDS-PAGE and ligand blot procedures.

Deglycosylation of IGFBPs

Serum (1 µL) and pituitary homogenates (25 µg of protein equivalent) were subjected to N-linked deglycosylation *(27)* to provide information on differences in sizes of IGFBPs. Briefly, samples were incubated overnight at 37°C in the presence or absence of 0.4 U of recombinant N-Glycosidase F (Boehringer Mannheim) in 20–25 µL of digestion buffer (0.6% Triton X-100; 0.25 *M* $NaPO_4$, 10 m*M* EDTA, pH 7.5). Samples were then mixed with equivalent volumes of gel-loading buffer and subjected to SDS-PAGE and ligand blot analysis as already described.

Northern Blot Analysis

Isolation and Northern blot analysis of total RNA were performed as previously reported *(19)*. In brief, anterior pituitary gland tissue was homogenized in guanidinium thiocyanate, and total RNA was purified through a CsCl gradient. Samples (15 µg of total RNA; minimum of five samples for each IGFBP) were denatured, separated by electrophoresis through an agarose-formaldehyde gel, and transferred to a nylon membrane. IGFBP-5 mRNA was then detected by hybridization with a (^{32}P)-UTP-labeled cRNA antisense probe transcribed from cDNA templates for rat IGFBP-2 and IGFBP-5 *(29)* or bovine IGFBP-3 *(19)*.

Statistical Analyses

All data were analyzed by analysis of variance using SAS general linear models procedures *(30)*. When a fixed effect was significant ($p < 0.05$), least squares means for response variables were compared across groups by the SAS least significant differences procedure. Differences among IGFBP profiles observed in serum, pituitary homogenates, and conditioned medium from pituitary cultures were evaluated using a randomized block design with cow as the blocking factor and sample type as a fixed effect. Response variables analyzed were migration rates of proteins (i.e., location of bands on gels as determined by scanning densitometry) and percentage of total IGF binding activity accounted for by individual IGFBPs.

The effects of in vitro treatment on IGFBP activity and concentrations of LH and FSH in conditioned medium and pituitary homogenates were evaluated by a model that included cow as a blocking factor and treatment as a fixed effect. Prior to analysis, data for IGFBPs and gonadotropin concentration in conditioned medium were divided by values obtained during the second 40-min incubation (conducted without hormone treatment) to adjust for unequal secretion rates owing to differences between tissue slices.

The effects of time after $PGF_{2\alpha}$ on IGFBP in pituitary homogenates were evaluated using gel ($n = 2$) as the blocking factor and day after $PGF_{2\alpha}$ as a fixed effect. The effects of time after $PGF_{2\alpha}$ on CL weight, circulating levels of progesterone, and concentrations of estradiol in the dominant follicle were evaluated using a completely random design. Simple correlations of IGFBP activities in pituitary tissue with luteal weight, circulating progesterone, and follicular fluid estradiol concentrations were obtained using the SAS program *(30)*.

Acknowledgments

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