

Hormonal Regulation of Insulin-Like Growth Factor Binding Protein Secretion by a Bovine Mammary Epithelial Cell Line

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ABSTRACT: A mammary epithelial cell line (MAC-T) established as a model for lactation was utilized to identify and characterize effects of various hormones upon insulin-like growth factor binding protein secretion. Ligand and immunoblot analyses of conditioned media indicated that insulin-like growth factor binding protein-2 was secreted by MAC-T cells. Insulin-like growth factor-I stimulated insulin-like growth factor binding protein-2 secretion in a dose-dependent manner, but prolactin and bovine somatotropin did not alter insulin-like growth factor binding protein-2 secretion. Insulin increased and cortisol decreased insulin-like growth factor binding protein-2 secretion. Effects of insulin-like growth factor-I

on insulin-like growth factor binding protein-2 secretion support previous studies using primary cultures of bovine mammary cells and bovine fibroblasts. Effects of cortisol and insulin on insulin-like growth factor binding protein-2 secretion may be explained by changes in protein synthesis. In addition, supraphysiological doses of insulin can cross-react with the insulin-like growth factor-I receptor and stimulate insulin-like growth factor binding protein-2 secretion. MAC-T cells provide a model system to study mechanisms that regulate local insulin-like growth factor-I bioactivity.

(Key Words: Insulin-Like Growth Factor Binding Protein, Mammary Cell-Line)

INTRODUCTION

Bovine somatotropin (bST) has been shown to increase milk production in lactating dairy cows (Peel and Bauman, 1987). The inability to detect high affinity bST receptors on mammary gland cell membrane fractions (Akers, 1985) have led to development of the hypothesis that bST does not act directly upon the mammary gland. Rather, bST may stimulate milk production by increasing insulin-like growth factor-I (IGF-I) secretion by the liver. Presence of IGF-I receptors in bovine mammary tissue (Dehoff et al., 1988) and elevated plasma IGF-I concentrations after exogenous bST administration (Davis, et al., 1987) support this hypothesis. Further, infusions of IGF-I into the arterial blood supply leading to the udders of lactating goats stimulated milk production (Prosser, et al., 1988). Since IGF-I mediates the actions of somatotropin in various tissues (Lowe, 1991), it is

considered likely that IGF-I also directly influences bovine mammary tissue function.

Meanwhile, insulin-like growth factor binding proteins (IGFBP) are involved in regulating the bioactivity and transport of IGF-I (Jones and Clemmons, 1995). Insulin-like growth factor binding proteins have been detected in sera from various animals, including dairy cows (Ronge and Blum, 1989). Vicini et al. (1991) observed an increase in the level of insulin-like growth factor binding protein-3 (IGFBP-3), the major circulating IGF binding protein, while the level of IGFBP-2 decreased when dairy cows were treated with bST. In dairy cows, it has been established that IGFBP-3 is bST-dependent (Cohick et al., 1992), although the mechanisms through which bST acts to alter IGFBP-2 levels are poorly understood. Studies have demonstrated that IGFBP are produced by a variety of cell types and act locally to modulate IGF-I activity (Baxter et al., 1989). Local production of IGFBP by mammary tissue (Capbell et al., 1991) may provide a mechanism through which IGF-I actions are regulated during lactation. *In vitro* studies focused on hormonal regulation of local IGFBP production by bovine mammary cells are limited due to complications in isolating and maintaining primary cultures of bovine

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mammary cells. To circumvent this problem, a bovine mammary epithelial cell-line (MAC-T), established as a model for lactation (Huynh et al., 1991) because they secrete milk proteins (Hanigan et al., 1993) and synthesize fatty acids (Kim et al., 1993), was used in the present study. The primary objectives of this study were to identify which forms of IGFBP are produced under defined conditions and determine effects of various hormones on IGFBP secretion by MAC-T cells.

MATERIALS AND METHODS

Cell culture

Bovine mammary epithelial cells (provided by J. D. Turner, McGill University, Canada) were seeded (2×10^5 cells) in 75-cm² culture flasks (Corning Inc., Corning, NY) containing Medium 199 (M-199; GIBCO/BRL Inc., Grand Island, NY) supplemented with 100 IU/ml penicillin; 100 µg/ml streptomycin (Mediatech Inc., Washington D.C.), 5% fetal calf serum (FCS; HyClone Inc., Logan, UT), 1 µg/ml cortisol (CORT; Sigma, St. Louis, MO), 1 µg/ml ovine prolactin (PRL; Sigma) and 1 µg/ml insulin (INS; Sigma). At confluence, MAC-T cells were trypsinized and transferred to 35 mm² or 100 mm² culture plates (Becton Dickinson, Lincoln Park, NJ) coated with extracellular matrix from mammary glands of a Holstein cow, as described below. Cells were plated confluent and allowed to attach to the extracellular matrix for 6 h, washed 3 times with M-199 and maintained in M-199 medium supplemented with 50 ng/ml INS and .5% gelatin for 20 h at 37°C in 5% CO₂ prior to use in experiments. Throughout the experimental periods media were replaced every 24 h.

Experiment 1 was conducted to determine whether or not IGF-1 and INS stimulates IGFBP secretion by MAC-T cells in a dose-dependent fashion. Cells were incubated with 0, 25, 50, 100, 200, and 400 ng/ml of human recombinant IGF-I (Collaborative Research Inc., Bedford, MA) or 0, 10, 100, 1,000 ng/ml of INS. Experiment 2 was conducted to determine the effects and interactions of various hormones on IGFBP secretion in MAC-T cells. Cells were incubated in M-199 supplemented with the following hormone(s) (1 µg/ml per hormone) for 48 h: 1) none; 2) CORT alone; 3) PRL alone; 4) INS alone; 5) CORT + PRL; 6) INS + PRL; 7) CORT + INS; 8) CORT + PRL + INS; and 9) bST alone (Monsanto Co., St. Louis, MO). After 48 h, protease inhibitors (.5 µg/ml leupeptin and 2 µg/ml aprotinin; Sigma) were added to samples of conditioned medium and stored at -20°C until analyzed for IGFBP content. Matrix and MAC-T cells were washed 3 times with phosphate-buffered saline plus 1 mM EDTA

and stored at -20°C. Cellular DNA concentrations were determined by the fluorimetric method of Labarca and Paigen (Labarca and Paigen, 1980).

Extracellular matrix preparation

The extracellular matrix for the cell culture system was prepared essentially as described by Wicha et al. (1982). Approximately 1 kg of mammary tissue was excised from the rear quarter of a lactating Holstein cow and homogenized in 10 volumes of water containing trypsin inhibitors (1 µg/ml leupeptin and 10 µg/ml soybean trypsin inhibitor; Sigma Chemical, St. Louis, MO) at 4°C. A Brinkmann Polytron homogenizer (at setting 7; Brinkmann Inc., Westbury, NY) was used until the tissue was finely minced (10-15 min). Homogenates were centrifuged at $1,000 \times g$ for 5 min and pellets were agitated overnight in 1 M NaCl plus protease inhibitors at 4°C. After centrifugation, pellets were washed 3 times with equal volumes of water and then with butanol/diethyl ether (2/3, vol/vol) on a shaker for 2 h at room temperature. Insoluble material was digested with 25 µg/ml DNase (Sigma) and 100 µg/ml RNase (Boehringer Mannheim Biochemical, Indianapolis, IN) for 4 h at 37°C in 5 volumes of M-199 with continuous stirring. After centrifugation, the matrix was washed 3 times with phosphate-buffered saline and stored in 3 volumes (wt/vol) of M-199 at -20°C. Extracellular matrix was plated using 2 ml of matrix suspension per 100 mm plate. The matrix-coated plate was incubated overnight in 5 ml of M-199 prior to cell plating.

Detection of IGFBP

Ligand blotting of IGFBP was conducted as described by Hossenlopp et al. (Hossenlopp et al., 1986). Conditioned media and dairy cow plasma samples were electrophoresed on a 12.5% discontinuous sodium dodecyl sulfate-polyacrylamide gel under nonreducing conditions using the Laemmli buffer system (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes via electroblotting under constant current (.4 Amp) at 15°C for 4 h. Blots were incubated overnight with 400,000 cpm/ml [¹²⁵I] IGF-I (Hodgkinson, 1989) and washed in Tris-buffered saline. Autoradiography was performed and relative band intensities, expressed as densitometric units (U), were quantified using a laser scanning densitometer (LKB, Inc., Piscataway, NJ).

Immunoblotting was performed as described by Liu et al., (Liu et al., 1993). Nitrocellulose membranes were first treated with 1% casein-Tris-buffered saline with .1% Tween-20 (TBST) buffer for 1 h at room temperature. This was followed by incubation with diluted antisera in

casein-TBST buffer overnight at 4°C. The IGFBP-2 antiserum was used at 1:400 dilution. The membranes were washed three times with TBST buffer and then incubated for 2 h at 28°C with alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (Calbiochem, San Diego, CA) diluted 1:5,000 in casein-TBST buffer. The membranes were washed three times with TBST buffer, after which they were incubated in alkaline phosphatase substrate [150 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (Calbiochem) and 300 µg/ml nitro blue tetrazolium (Calbiochem) in 100 mM Tris and 1 mM EDTA, pH 2.9] for 5 min, rinsed in water, and allowed to air dry in the dark.

The IGFBP-2 antibody was prepared as described (Liu et al., 1993). It was raised against a synthetic fragment of rat IGFBP-2. The fragment used was derived from the unique hydrophobic region found within the midportion of the IGFBP-2 away from the 18 homologous cysteines. As demonstrated (Liu et al., 1993), the antibody reacts specifically with IGFBP-2 and shows no cross-reactivity with the other IGFBP. The molecular weight of IGFBP-2 was determined by comparison to molecular weight markers (Bio Rad, Inc., Hercules, CA).

Statistical analysis

The statistical model was based on a one way classification analysis of variance with treatment groups that consisted of hormone or hormone concentration as the main effect. Tests of significance for all possible comparisons were determined by Student Newman-Keuls Test in PROC GLM ANOVA (SAS, 1989). Data are expressed as means + standard errors of means.

RESULTS

Immunoblotting of conditioned medium with antiserum specific for IGFBP-2 resulted in a single band at 34 kDa (figure 1). Ligand blotting analyses of cell-conditioned medium demonstrated that MAC-T cells synthesized and secreted a 34 kDa IGFBP (figure 1).

Insulin-like growth factor-I treatment increased ($p < 0.05$) IGFBP-2 secretion as compared to untreated MAC-T cells (figure 2). The stimulatory effect of IGF-I on IGFBP-2 secretion occurred in a dose-dependent fashion up to 100 ng/ml of IGF-I. Insulin concentrations (100 and 1,000 ng/ml) increased ($p < 0.05$) IGFBP-2 secretion as compared to treated (10 ng/ml INS) or untreated MAC-T cells (figure 3). MAC-T cells treated with 1,000 ng/ml INS secreted higher ($p < 0.05$) levels of IGFBP-2 as compared to MAC-T cells treated with 100 ng/ml INS.

Altered levels of IGFBP-2 by different hormones were

presented in figure 4. Secretion of IGFBP-2 was decreased ($p < 0.05$) in MAC-T cells maintained in media supplemented with CORT or CORT + PRL as compared to untreated MAC-T cells. Conversely, INS alone or INS + PRL resulted in maximal levels of IGFBP-2 secretion as compared to all other treatment groups ($p < 0.05$). MAC-T cells treated with CORT + INS or CORT +

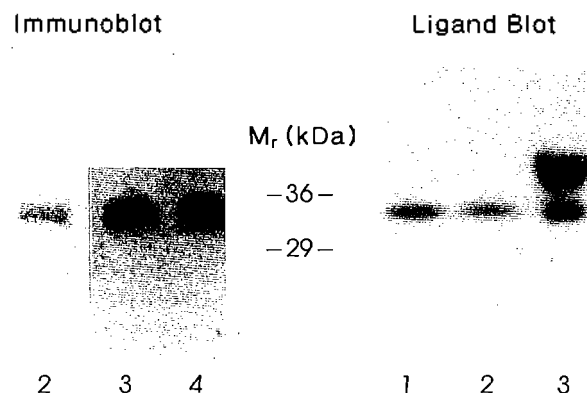


Figure 1. Detection of insulin-like growth factor binding protein-2 secreted by MAC-T cells using immunoblot and ligand blot analysis. Cells were cultured for 48 h on mammary extracellular matrix in serum free media containing 1 µg/ml cortisol, 1 µg/ml prolactin and 1 µg/ml insulin. Conditioned medium (lane 1 and 2) and dairy cow plasma (lane 3 and 4) were immunoblotted with rat IGFBP-2 antisera and ligand blotted with [¹²⁵I]-IGF-I.

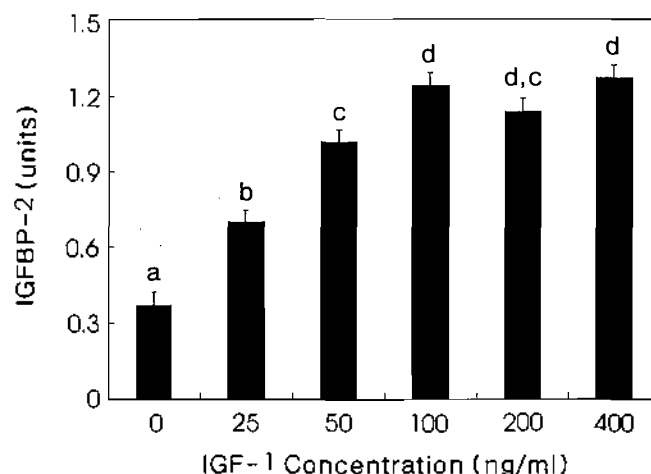


Figure 2. Effects of insulin-like growth factor-I on insulin-like growth factor binding protein-2 secretion by MAC-T cells. Cells were cultured for 48 h on mammary extracellular matrix in serum free media containing 0, 25, 50, 100, 200 or 400 ng/ml of IGF-I. IGFBP-2 is expressed as densitometric units (Units). Means with different letters within a hormone treatment are significantly different ($p < 0.05$), $n=3$ /treatment group.

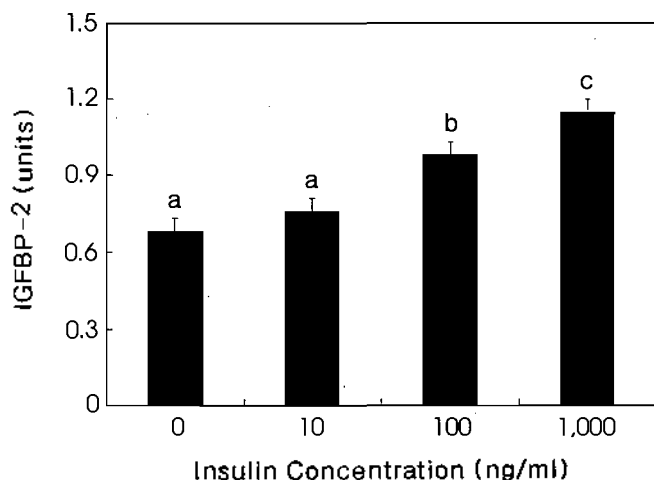


Figure 3. Effects of insulin on insulin-like growth factor binding protein-2 secretion by MAC-T Cells. Cells were cultured for 48 h on mammary extracellular matrix in serum free media containing 0, 10, 100 or 1,000 ng/ml of insulin. IGFBP-2 is expressed as densitometric units (Units). Means with different letters within a hormone treatment are significantly different ($p < 0.05$), $n=3$ /treatment group.

PRL + INS increased ($p < 0.05$) IGFBP-2 secretion relative to untreated MAC-T cells, but CORT + INS or CORT + PRL + INS treated MAC-T cells produced less ($p < 0.05$) IGFBP-2 as compared to INS or INS + PRL treated MAC-T cells. Neither PRL or bST had an effect on IGFBP-2 secretion by MAC-T cells.

DISCUSSION

The effects of bST on lactation in ruminants have been known for over fifty years (Asimov and Krouze, 1937). Previous studies have demonstrated that bST increases lactational performance in dairy cows (Peel and Bauman, 1987). The mechanisms of bST actions are complex and involve numerous events in several tissues. Ironically, bST does not appear to act directly upon mammary secretory cells due to the lack of detectable bST binding sites in bovine mammary tissue (Akers, 1985). The presence of IGF-I receptors on bovine mammary tissue (Dehoff et al., 1988) and elevated plasma IGF-I concentrations in response to bST injections (Davis et al., 1987; Cohick et al., 1989) have led to speculation that IGF-I may be a mediator of bST in stimulating milk production in the dairy cow.

If the speculation above is acceptable, one must concern about the implication of IGFBP with regard to the IGF-I actions since the bioactivity of IGF-I is influenced by local and systemic production of IGFBP (Jones and Clemmons, 1995). Recent studies have shown that bovine mammary cells can produce IGFBP (McGrath et al., 1991; Campbell et al., 1991), including MAC-T cells (Woodward et al., 1996) which may act locally to modulate IGF-I activity. In the present study, untreated MAC-T cells produced one species of IGFBP, immunologically detected as IGFBP-2 with a molecular weight of 34 kDa.

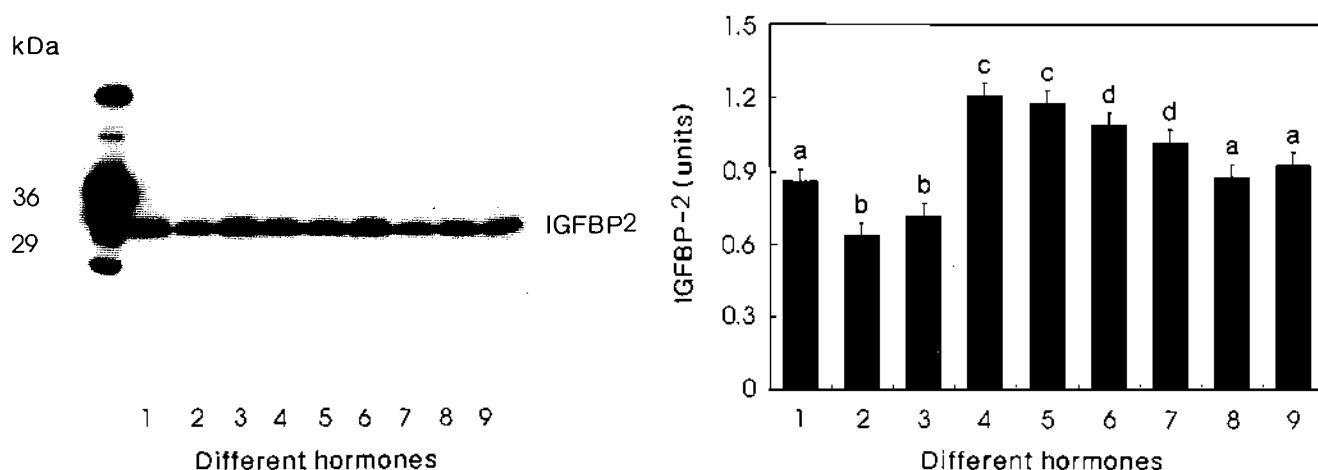


Figure 4. Hormonal effects on insulin-like growth factor binding protein-2 secretion by MAC-T Cells. Cells were cultured for 48 h on mammary extracellular matrix in serum free media supplemented with the following hormone (s) (1 μ g/ml per hormone); none (lane 1), cortisol (CORT, lane 2), prolactin (PRL, lane 3), insulin (INS, lane 4), CORT+PRL (lane 5), INS+PRL (lane 6), CORT+INS (lane 7), CORT+PRL+INS (lane 8) or bST (lane 9). IGFBP-2 is expressed as densitometric units (Units). Means with different letters within a hormone treatment are significantly different ($p < 0.05$), $n=3$ /treatment group. Top panel shows the detected levels of IGFBP by the ligand blot method.

Our result was in contrast to the four different species of IGFBP secreted by primary cultures of bovine mammary cells (McGrath et al., 1991). Several explanations may account for these differences. McGrath et al. (1991) demonstrated that two of the four IGFBP were not secreted by primary cultures of bovine mammary cells from mid-pregnant animals when FCS was removed from the incubation medium. Only IGFBP-2 and IGFBP-3 were secreted suggesting that some serum factor(s) is required for secretion of the 21 and 28 kDa IGFBP. Further, primary cultures of mammary cells, in contrast to MAC-T cells, contain many cell types that may secrete additional species of IGFBP. Cultured bovine fibroblasts secrete several species of IGFBP as detected by ligand blotting analysis (Conover, 1990). DeLeon et al. (1990) utilized several breast cancer cell lines to demonstrate that no single cell-line secreted all species of IGFBP.

In the present study, IGFBP-2 secretion into the media by MAC-T cells was increased by IGF-I in a dose-dependant manner. These observations are in agreement with those of McGrath et al. (McGrath et al., 1991), Conover et al. (1990) and Woodward et al. (1996) who reported that secretion of IGFBP-2 was dependant on IGF-I concentrations. The significance of IGF-stimulated IGFBP-2 secretion by MAC-T cells remains to be determined. However, based on reports that IGFBP-2 potentiates the activity of IGF-I (Bourner et al., 1992), one can speculate that a bST-induced rise in IGF-I levels may stimulate local secretion of IGFBP-2 and augment the effects of IGF-I on milk production. In contrast, serum and lymph levels of IGFBP-2 in lactating dairy cows are depressed by bST administration (Vicini et al., 1991; Cohick et al., 1992). Several hypotheses may explain this discrepancy between *in vivo* and *in vitro* observations of IGFBP-2 regulation. Alterations in circulating IGFBP-2 levels are not necessarily indicative of local tissue IGFBP regulation. Albiston and Herington (Albiston and Herington, 1992) demonstrated that tissue expression of IGFBP is regulated differently in different tissues. Moreover, other systemic or local factors may be involved in antagonizing or stimulating IGFBP-2 production. Cohick et al. (Cohick et al., 1992) speculated that bST may antagonize the effects of IGF-I on IGFBP-2 levels in lactating dairy cows. In support, IGF-I infusion increased IGFBP-2 levels in humans but had no effect on IGFBP-2 levels when the subjects were treated with somatotropin (Zapf et al., 1990). Further, transgenic mice deficient in somatotropin have elevated IGFBP-2 levels as compared to non-transgenic mice (Camacho-Hubner et al., 1991).

Bovine somatotropin may indirectly influence IGFBP-2 secretion by altering concentrations of other hormones during lactation which act upon the mammary gland. Insulin has been suggested as a negative regulator of IGFBP-2 in normal and diabetic rodents (Boni-Schnetzler et al., 1990; Ooi et al., 1990). Observations in bST-treated dairy cows during late lactation and the dry period are consistent with this hypothesis but not during early lactation when cows are in negative energy balance (Vicini et al., 1991). The present and previous studies using normal bovine mammary cells (McGrath et al., 1991), bovine fibroblasts (Conover, 1990) or bovine epithelial cells (Cohick and Clemmons, 1991; Underwood et al., 1996) do not support the hypothesis that insulin is a negative regulator of IGFBP-2 production. In fact, supraphysiological concentrations of insulin stimulate IGFBP-2 secretion by MAC-T cells. This observation suggests that the effect of insulin may be mediated via IGF receptors since, at high concentrations, insulin can bind to IGF receptors with approximately 1% the affinity of IGF-I (Clemmons and Underwood, 1991).

Previous studies suggest that prolactin and glucocorticoids are primary promoters of lactogenesis in ruminants (Akers, 1985). The importance of cortisol and prolactin for milk protein production in bovine mammary organ cultures have been previously demonstrated (Hodgkinson et al., 1989). At present, the influences of these hormones on circulating and tissue IGFBP levels in the intact animal are unknown. Cortisol with or without prolactin decreased IGFBP-2 secretion by MAC-T cells, which may be due to an overall catabolic effect on protein synthesis. Prolactin did not have a major effect on IGFBP-2 secretion by MAC-T cells unless the hormone was co-incubated with supraphysiological levels of insulin. The insulin effect was probably due to cross-reactivity with the IGF receptor. Campbell et al. (Campbell et al., 1991) observed that addition of prolactin and physiological levels of insulin did not alter IGFBP secretion by mammary explants. These studies support the observation that prolactin and physiological concentrations of insulin do not influence IGFBP-2 secretion by MAC-T cells, and also suggest that IGFBP secretion is involved not only with IGF-I but also with a variety of other hormones.

In overall conclusion, the mammary gland is constantly influenced by a variety of effectors from circulation and cells. While the importance and contribution of circulating IGF-I and IGFBP cannot be ignored, local secretion of these factors and modulators directly influence the mammary gland. Local levels are presumably composed of several species of IGFBP from

circulation and tissue secreted IGFBP that provide a mechanism to modulate the bioactivity of IGF-I. In the present study, IGFBP-2 was identified from bovine mammary epithelial cells (MAC-T) and secretion was regulated by IGF-I and other effectors. Integration of this information can be difficult without knowledge of other effectors that may regulate IGF-I and all species of IGFBP produced by the tissue. Further studies to examine the effects of bST on mammary gland IGF-I and IGFBP expression in dairy cows during various stages of lactation are necessary to develop an accurate understanding of the local mechanisms that regulate the actions of IGF-I and milk production.

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