NUTRITIONAL REGULATION OF SERUM INSULIN-LIKE GROWTH FACTOR-I CONCENTRATION IN CATTLE

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ABSTRACT

The overall objective of this dissertation research was to understand the mechanisms by which serum insulin-like growth factor-I (IGF-I) is regulated by nutritional intake in cattle. Two studies were conducted to achieve this objective. In the first study, effects of feeding levels on basal and growth hormone (GH)-stimulated serum concentrations of IGF-I, IGF binding protein-3 (IGFBP-3) and acid-labile subunit (ALS), and their mRNA expression in the liver were determined in beef cows. It was found that increased nutritional intake did not alter basal concentrations of serum IGF-I, IGFBP-3 or ALS, or their mRNA expression in the liver. However, under increased nutritional intake, GH administration stimulated a greater increase in serum IGF-I concentration, and this greater increase was not due to reduced degradation of IGF-I in serum. Increased nutritional intake did not enhance GH-stimulated IGF-I mRNA expression in the liver, but it increased the amount of IGF-I mRNA associated with polysomes, suggesting that liver translation of IGF-I mRNA is enhanced under increased nutritional intake. Under increased nutritional intake, GH also stimulated greater increases in serum IGFBP-3 and ALS concentrations, but these greater increases were not due to greater expression or translation of their mRNAs in the liver. Taken together, these results suggest that translation of GH-stimulated IGF-I mRNA in the liver is enhanced under increased nutritional intake and this enhancement may be partially responsible for the greater GH-stimulated increase in serum IGF-I concentration. These results also suggest that the greater GH-stimulated increases in serum IGFBP-3 and ALS may be secondary to the greater increase in serum IGF-I because increased IGF-I may increase

the formation of IGF-I/IGFBP-3/ALS complexes, thereby increasing the retention of IGFBP-3 and ALS in the blood. In the second study, the effects of food deprivation on serum IGF-I concentration in steers and the underlying mechanism were determined. It was found that food deprivation decreased serum IGF-I concentration and that this decrease was not due to increased IGF-I degradation in serum. Food deprivation decreased liver IGF-I mRNA expression, and this decrease was associated with decreased expression of GH receptor (GHR) mRNA and protein in the liver. Food deprivation was also associated with increased mRNA expression of two inhibitors of the GHR signaling pathway, suppressor of cytokine signaling-2 (SOCS2) and cytokine-inducible SH2 protein (CIS). These results suggest that decreased IGF-I gene expression in the liver may be at least partially responsible for the decrease in circulating IGF-I concentration during food deprivation, and that the former decrease may be due to increased expression of SOCS2 and CIS, and decreased expression of GHR in the liver. Overall, this dissertation research indicates that multiple mechanisms are involved in nutritional regulation of circulating IGF-I concentration in cattle.

Key Words: Nutrition, Insulin-like growth factor-I, Growth hormone, IGF binding protein-3, Acid-labile subunit, Cattle

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Introduction

Insulin-like growth factor-I (IGF-I) is an important regulator with anabolic properties for somatic growth and cellular metabolism. Circulating IGF-I is primarily synthesized in the liver, in which IGF-I gene expression is regulated by growth hormone (GH) and nutritional intake. Food deprivation or restriction decreases serum IGF-I concentration, whereas increased nutritional intake increases serum IGF-I concentration response to GH administration. Nutritional regulation of serum IGF-I theoretically can be mediated by at least four mechanisms: changed IGF-I gene expression in the liver, changed IGF-I degradation in serum, changed IGF-I clearance from serum and changed IGF-I gene expression in extrahepatic tissues. The two studies reported in this dissertation were conducted to investigate these mechanisms in cattle. In the first study, the involvement of these mechanisms in mediating the effects of different feeding levels on basal and GH-stimulated circulating IGF-I concentration was determined in cows. In the second study, the involvement of these mechanisms in mediating the effects of food deprivation on circulating IGF-I concentration was determined in steers.

Chapter I

Review of Literature

Introduction

Insulin-like growth factor-I (IGF-I), also called somatomedin C, is a 70-amino acid polypeptide hormone with anabolic properties for somatic growth and cellular metabolism. In the blood, the majority of IGF-I circulates as a binary complex with IGF binding protein-3 (IGFBP-3) or a ternary complex with IGFBP-3 and acid-labile subunit (ALS). Both IGFBP-3 and ALS regulate IGF-I transport to target tissues and its retention in the circulation. Circulating IGF-I is primarily synthesized in and secreted from the liver. Hepatic IGF-I gene expression is regulated by growth hormone (GH), which is a 191-amino acid pituitary hormone. Growth hormone stimulates IGF-I gene expression through a membrane receptor, growth hormone receptor (GHR), and the signaling pathways from GHR. The IGF-I gene expression is also regulated by nutritional intake. This review will concentrate on GH- and nutritional regulation of circulating IGF-I system, including IGF-I, IGFBP-3 and ALS.

IGF-I

IGF-I gene and biosynthesis

IGF-I is synthesized in many tissues, including liver, brain, skeletal muscle, and heart (Kajimoto and Rotwein, 1989). The IGF-I mRNA can be detected in most tissues before birth, but the expression is greatly increased in liver and decreased elsewhere after birth. Liver IGF-I expression is increased through puberty and declined slowly with aging (Dickson et al., 1991; Yu et al., 1999). Liver-derived IGF-I contributes to about 75% of circulating IGF-I (Sjogren et al., 1999).

The IGF-I gene consists of six exons and five introns (Kajimoto and Rotwein, 1989). There are two leader exons in the IGF-I gene, exons 1 and 2. Transcription of IGF-I gene can be initiated from both of them, producing two classes of IGF-I mRNA: class 1 IGF-I mRNA containing exon 1 and class 2 IGF-I mRNA containing exon 2 (Rotwein et al., 1987; Tobin et al., 1990; Dickson et al., 1991; Pell et al., 1993). Transcription of IGF-I gene in the liver is largely initiated from exon 1, and thus class 1 IGF-I mRNA is more abundant than class 2 in the liver (Ohlsen et al., 1993; Weller et al., 1993; Wang et al., 2003; Ohtsuki et al., 2007). Class 1 and class 2 IGF-I mRNAs differ in stability and translatability, but they encode the same mature IGF-I protein (Rotwein et al., 1987; Yang et al., 1995).

Serum IGF-I and liver IGF-I mRNA in cattle

Both circulating IGF-I concentration and liver IGF-I mRNA expression in cattle vary with breeds, sex, age and physiological conditions. The lowest serum IGF-I concentration (~39 ng/mL) in female cattle was found in postparturient Holstein cow, and the highest (~157 ng/mL) was in the growing beef heifers. However, the lowest and highest serum IGF-I concentrations in male cattle were in 1-day-old Holstein calves (~63 ng/mL) and 16-month-old Simmental-type bulls (~516 ng/mL), respectively (Nikolic et al., 2001). Serum IGF-I concentrations in dairy cows were lowest after 24 h of post-partum (~45 ng/mL), and then increased and finally maintained at a higher level throughout the 8 mo of lactations (~90 ng/mL). Serum IGF-I concentrations was further increased during the dry period (~110 ng/mL) (Abribat et al., 1990). Hepatic IGF-I mRNA was increased after birth and was 4 to 8 times higher in veal calves, fattened castrated bulls and intact bulls than in 8-d old calves (Cordano et al., 2000). Liver IGF-I mRNA and serum IGF-I were higher in bulls than steers or heifers, while skeletal muscle IGF-I mRNA was not different (Hannon et al., 1991).

IGF-I function

IGF-I is important for somatic growth and cellular metabolism. Liver IGF-I mRNA, liver IGF-I production, and plasma IGF-I were correlated with weight gain in bulls, steers or heifers (Hannon et al., 1991). Positive residual correlation was found in beef cattle between IGF-I and average daily gain (ADG) and between IGF-I and feed efficiency (Stick et al., 1998). Plasma immunoreactive IGF-I was found to correlate well with the whole body protein synthesis rate (Nam et al., 1990).

Recombinant IGF-I administration or genetic manipulation of IGF-I gene provides a powerful tool to study the biological functions of IGF-I. The IGF-I treatment increased cellular uptake of amino acids and glucose from plasma, inhibited proteolysis and hepatic gluconeogenesis, and accelerated protein synthesis in the muscle (Boulware et al., 1992; Giordano et al., 1995). In GH-deficient (GHD) animals that are dwarf, administration of IGF-I resulted in significant increases in body length and weight gain, and these increases were not due to food or water intake which was not changed (Gargosky et al., 1994; van Buul-Offers et al., 1995).

With global deletion of IGF-I, fetal growth was markedly decreased, postnatal growth was impaired and adults were infertile (Liu et al., 1993). Liver IGF-I deficient (LID) mice had a 75% reduction in circulating IGF-I concentration, a corresponding fourfold increase in GH level, a reduction in cortical radial bone growth and axial skeletal growth, a mild reduction in bone size and a marked reduction in femoral volumetric bone density, and insulin resistance in muscle, liver and fat tissues (Sjogren et al., 1999; Yakar et al., 2002a; Haluzik et al., 2003). Therefore, decreasing circulating IGF-I may inhibit cellular anabolism.

Mice with liver-specific overexpression of IGF-I had longer body length and heavier body weight with a 50-60% increase in serum IGF-I and a decrease in serum GH. The transgenic mice had more and thicker skeletal muscle fibers resulting in a heavier lean mass than wild-type (WT) mice (Liao et al., 2006). Therefore, increasing serum IGF-I can stimulate both somatic growth and lean mass. Overexpression of IGF-I in extra-hepatic tissues causes tissue-specific overgrowth, despite no difference in circulating IGF-I level (Coleman et al., 1995; Weber et al., 1998; Zhao et al., 2000). Muscle-specific overexpression of IGF-I in mice caused a 47-fold increase of IGF-I concentrations in skeletal muscle and myofiber hypertrophy (Coleman et al., 1995). Osteoblast-specific overexpression of IGF-I increased bone formation rate, bone mineral density, femoral cancellous bone volume and osteocyte lacunea occupancy in mice (Zhao et al., 2000).

IGF-I receptor signaling pathways

At the cellular level, the functions of IGF-I are mediated by its receptors. There are three classes of receptors that can bind IGF-I. The type 1 IGF receptor (IGF-IR) binds IGF-I with high affinity, IGF-II to a cross-reactivity of approximately 40%, and insulin only when it is in high concentrations (Maly and Luthi, 1986; Liu et al., 1993). The type 2 IGF receptor (IGF-IIR) binds to IGF-II with high specificity and IGF-I with low affinity, but has no affinity for insulin (Taylor et al., 1987). The insulin receptor (IR) binds to IGF-I with roughly 100-fold lower affinity than insulin. However, in adipocytes IGF-I is predominantly mediated by IR because of the absence of IGF-IR, and IR can partly mediate the function of IGF-II on mouse embryogenesis (Louvi et al., 1997).

The IGF-IR is a transmembrane glycoprotein and consists of two alpha subunits, which bind to IGF-I, and two beta subunits, which possess intrinsic tyrosine kinase activity and are

approximately 84% homology with the insulin receptor (Jacobs et al., 1983; Ullrich et al., 1986). The IGF-IR is essential for postnatal life, and the IGF-I receptor gene-deleted mouse died at birth (Butler and LeRoith, 2001). Binding of IGF-I to IGF-IR leads to activation of the tyrosine kinase, which phosphorylates the insulin receptor substrate-1 (IRS-1) (Jacobs et al., 1983; Rubin et al., 1983). Tyrosine-phosphorylated IRS-1 interacts with cytoplasmic proteins with src homology 2 (SH2) domains, such as phosphatidylinositol 3-kinase (PI3K) and phosphotyrosine phosphatase SHP-2 (Syp) (Giorgetti et al., 1993; Kuhne et al., 1993; Lee et al., 1993). These molecules then activate several signaling cascades, leading to transcriptional or translational changes in targeted genes that are involved in cellular metabolism, proliferation, apoptosis (Adams et al., 2000; Yanochko and Eckhart, 2006).

Circulating IGF-I system

Circulating IGF-I primarily comes from liver

Liver-specific IGF-I gene inactivation provides direct evidence that the primary source of circulating IGF-I is liver. In mice with liver-specific IGF-I gene inactivation, circulating IGF-I concentration was 75% lower than in wild-type (WT) mice (Sjogren et al., 1999). Liver-specific overexpression of IGF-I in mice increased serum IGF-I by 50-60% (Liao et al., 2006). However, overexpression of IGF-I in extra-hepatic tissues, such as muscle, mammary gland and bone, did not change circulating IGF-I level (Coleman et al., 1995; Weber et al., 1998; Zhao et al., 2000).

There is a positive correlation between hepatic IGF-I mRNA and plasma IGF-I concentrations in neonatal calves, veal calves, fattened castrated bulls and mature intact bulls (Cordano et al., 2000), indicating hepatic IGF-I production may also contribute to circulating IGF-I in cattle.

IGF-I mainly circulates as a binary or a ternary complex

In the bloodstream, IGF-I forms a heterodimeric or heterotrimeric complex with IGF binding proteins, termed IGFBP-1 to IGFBP-6, and acid-labile subunit (ALS). There is less than 5% of total IGF-I circulating as free IGF-I in the postnatal blood (Frystyk et al., 1999; Shimizu et al., 1999; Yu et al., 1999), and most of serum IGF-I is bound to one of the IGF binding proteins, mostly to IGFBP-3 (Albiston and Herington, 1990; Baxter et al., 2002; Yakar et al., 2002b; Nindl et al., 2003). Approximately 20% of IGF-I circulates as a 40-50 kDa binary complex with IGFBP-3. The majority of blood IGF-I (~80%) circulates in a 150 kDa ternary complex that also contains IGFBP-3 and ALS (Conover et al., 1990; Boisclair et al., 2001; Nindl et al., 2003).

Ternary complex increases the retention time of IGF-I, IGFBP-3 and ALS in blood

The availability of endocrine IGF-I for target tissues is dependent on the retention time of IGF-I in the bloodstream. The retention time of free IGF-I is significantly shorter than bound IGF-I in the bloodstream, and association into the ternary complex significantly increases the half-life of serum IGF-I from approximately 20 min in the free form to more than 12 h in the ternary complex (Francis et al., 1988; Guler et al., 1989). The retention time of IGFBP-3 is also greatly shorter when administrated alone than when coadministrated with IGF-I (Lewitt et al., 1994; Lee et al., 1997; Firth et al., 2002).

The retention of IGF-I or other component of the ternary complex is dependent on their ability to cross the capillary barrier. Binary complex rapidly crosses capillary endothelia (Arany et al., 1993), whereas the 150 kDa IGF-I / IGFBP-3 / ALS complex can not cross the capillary barrier to extravascular compartments or cross in very little proportion (Binoux and Hossenlopp, 1988). The bioavailability of IGF-I can be regulated by the formation and dissociation of the ternary complex (Holman and Baxter, 1996). It is believed that the binary complex of IGF-I /

IGFBP-3 functions as a vehicle for IGF-I to be delivered to the extravascular tissues, whereas the ternary complex functions as a reservoir for circulating IGF-I and decreases the clearance of IGF-I from the blood (Binoux and Hossenlopp, 1988; Yakar et al., 2002b).

Growth hormone regulation of circulating IGF-I system

GH regulation of IGF-I gene expression

In hypophysectomized animals in which pituitary GH is deficient (GHD), serum IGF-I concentrations are up to 15-fold lower than those in pituitary-intact animals (Mathews et al., 1986; Isgaard et al., 1989; Iida et al., 2004; van Dijk et al., 2006). Administration of GH to the GHD animals increases liver IGF-I mRNA expression and restores serum IGF-I to its normal level (Mathews et al., 1986; Isgaard et al., 1989; Bichell et al., 1992; Iida et al., 2004). Serum IGF-I concentrations in lactating cows were increased by administration of sustained-release recombinant bovine GH, and the increase is dose dependent (Bilby et al., 1999). These results suggest IGF-I gene expression in liver is regulated by GH.

The effect of GH on IGF-I gene expression is rapid. Hepatic IGF-I mRNA was significantly increased within 2 h of GH treatment, and reached the peak level after 4 h of injection. However, the increase in nuclear nascent IGF-I mRNA was observed within 30 min of GH treatment, and reached the peak level after 2 h of administration (Bichell et al., 1992). These results indicate that GH stimulates IGF-I expression at the level of transcription.

Growth hormone receptor

At the cellular level, the regulatory effect of GH on its target cells is mediated through a transmembrane protein, the growth hormone receptor (GHR). The GHR is a single transmembrane protein of approximately 620 amino acids. The mature GHR has 3 major domains: an extracellular domain of about 246 amino acids at its N-terminus for GH-binding, a

hydrophobic transmembrane domain of 24 amino acids, and an intracellular domain of approximately 350 amino acids at its C-terminus for signaling transduction (Edens and Talamantes, 1998). The GHR transcripts have been detected in a variety of tissues including liver, muscle, kidney, lung, mammary gland, placenta, and adipose tissue, with the highest level of expression being in the liver (Tiong and Herington, 1991; Mertani and Morel, 1995; Jiang and Lucy, 2001). Nine GHR mRNA variants (1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, and 1I) have been identified in postnatal cattle, which differ in the leader exon (exon 1) and are identical in the nine coding exons (exon 2 to 10) (Edens and Talamantes, 1998). After translation, GHR is translocated to the plasma membrane. So the availability of GHR on cell surface is the primary determinant of GH responsiveness (Eshet et al., 1984; Liu et al., 1999; Coschigano et al., 2003). Liver IGF-I mRNA levels were strongly correlated with GHR mRNA levels in GH-transgenic, GH-antagonist transgenic, lit/lit mice and control littermate (Iida et al., 2004). Deletion of GHR gene, or GHR malfunction due to GHR mutation, results in lower serum IGF-I and severe postnatal growth retardation (Eshet et al., 1984; Rowland et al., 2005).

JAK2-STAT5 signaling pathway

Janus kinase 2 (JAK2) is a ubiquitously expressed cytoplasmic tyrosine kinase, which serves as a critical molecule in mediating signals generated by receptors in response to many cytokines and hormones, and performs catalytic function of transphosphorylation (Argetsinger et al., 1993). The binding of GH to GHR results in receptor dimerization which leads to phosphorylation of receptor-associated JAK2 and activation of JAK2 (Chow et al., 1996). The GHR and several signaling proteins, such as signal transducer and activator of transcription 5 (STAT5), are subsequently phosphorylated on key tyrosine residues by pJAK2, resulting in

changes in expression of GH-target genes, such as IGF-I (Argetsinger et al., 1993; Chow et al., 1996; Flores-Morales et al., 1998; von Laue et al., 2000).

STAT5 proteins are transcription factors that are essential for GH-induced liver IGF-I gene expression. Deletion or mutation of STAT5b results in severe growth hormone insensitivity, in which serum IGF-I concentration was unusually low and was not restored with GH treatment (Woelfle and Rotwein, 2004; Hwa et al., 2005). However, constitutively active STAT5b stimulated robust transcription of IGF-I in the pituitary-deficient rats (Woelfle and Rotwein, 2004). The STAT5 can be phosphorylated on the key tyrosine residues to form activated phospho-STAT5 (pSTAT5). pSTAT5 forms dimers, translocates into the nucleus, and then activates transcription of targeted genes (Flores-Morales et al., 1998; von Laue et al., 2000; Davey et al., 2001).

Negative regulatory factors in the GHR signaling pathway

The GHR signaling pathway is negatively regulated by the suppressors of cytokine signaling (SOCS), which include eight proteins, cytokine-inducible SH2 protein (CIS) and SOCS1 to 7. All of them contain two important domains, a SH2 domain and a SOCS-box, which can bind to other proteins containing SH2-binding site, such as cytokine receptor or JAKs and thereby reducing signal transduction or increasing the ubiquitin-mediated degradation of target proteins (Starr et al., 1997; Tan and Rabkin, 2005).

SOCS2 and CIS are two important negative regulators of GH signal transduction (Greenhalgh and Alexander, 2004). SOCS2 binds to the SH2-binding site of phosphorylated GHR and inhibits the phosphorylation and activation of STAT5b (Greenhalgh and Alexander, 2004). SOCS2 knockout does not elevate GH level in the transgenic mice, but causes gigantism, with more rapid growth after weaning (Greenhalgh et al., 2002). These mice weigh 40% more

than WT mice and have higher local IGF-I production resulting in the enlargement of organs (Metcalf et al., 2000). The STAT5 signaling in response to GH was moderately prolonged in the primary hepatocytes from SOCS2 knockout mice (Greenhalgh et al., 2002).

CIS was shown to mask the STAT5-binding sites of GHR resulting in the inhibition of STAT5 activation (Karlsson et al., 1999). Cell transfection studies indicated that overexpression of CIS attenuates STAT5 tyrosine-phosphorylation in Ba/F3 cells (Matsumoto et al., 1997), and CIS-overexpressed transgenic mice had lower body weight than WT mice (Matsumoto et al., 1999).

The expression of SOCS2 and CIS is STAT5b-dependent and is regulated by GH. Dominant-negative STAT5b inhibited the expression of SOCS2 and CIS in the liver of pituitary-deficient rats (Woelfle and Rotwein, 2004). The SOCS2 and CIS mRNAs were not detectable in the hypophysectomized rats, and increased after GH treatment (Karlsson et al., 1999), indicating that the expression of SOCS2 and CIS in the liver is dependent on the presence of GH.

GH regulation of IGFBP-3

Serum IGFBP-3 is regulated by GH. Growth hormone-deficient mice have a lower serum IGFBP-3 (Camacho-Hubner et al., 1991), whereas acromegalic patients have a higher serum IGFBP-3 (Fukuda et al., 2002). Growth hormone administration restores serum IGFBP-3 in GHD rats (Gargosky et al., 1994). The increase in serum IGFBP-3 after GH administration was found to be mainly in the form of ternary complex, whereas the noncomplex-associated IGFBP-3 fraction was not changed after treatment (Laursen et al., 2000).

STAT5b is a key regulator for liver IGFBP-3 expression. Absence of STAT5b or dominant-negative STAT5b decreases circulating IGFBP-3 and inhibits GH-stimulated

transcription of IGFBP-3. In contrast, constitutively active STAT5b stimulated transcription of IGFBP-3 in the absence of GH (Woelfle and Rotwein, 2004; Hwa et al., 2005), suggesting that GH regulates IGFBP-3 expression through STAT5b.

GH regulation of ALS

GH deficiency as a result of hypophysectomy (Chin et al., 1994; Fielder et al., 1996; Ooi et al., 1997) or mutation in the GH-releasing hormone receptor (Aguiar-Oliveira et al., 1999) causes great reductions in circulating ALS and hepatic ALS mRNA levels. Malfunction of GHR, such as Laron syndrome, is also associated with lower circulating ALS (Kanety et al., 1997). However, excess GH such as acromegaly has higher circulating ALS than normal (Juul et al., 1998; Stadler et al., 2001; Fukuda et al., 2002). Treatment with recombinant GH in GHD restored serum ALS and normal formation of the 150-kDa ternary complex within hours (Gargosky et al., 1994; Fielder et al., 1996; Labarta et al., 1997; Ooi et al., 1997; Stadler et al., 2001). These results strongly suggest that ALS gene expression is directly regulated by GH.

GH regulation of ALS expression is STAT5b-dependent. The STAT5b deficiency or dominant-negative STAT5b results in abnormally low expression of liver ALS and low circulating level of ALS, and GH can not restore ALS production in either case (Hwa et al., 2005). However, constitutively active STAT5b stimulates ALS transcription even in the absence of GH (Woelfle and Rotwein, 2004). A nine-base pair cis-element resembling an interferon-gamma-activated sequence-like element is presented in the ALS promoter, which is conserved in human and mouse. Binding of STAT5 to this element may mediate GH activation of ALS gene expression (Ooi et al., 1998; Boisclair et al., 2000; Suwanichkul et al., 2000).

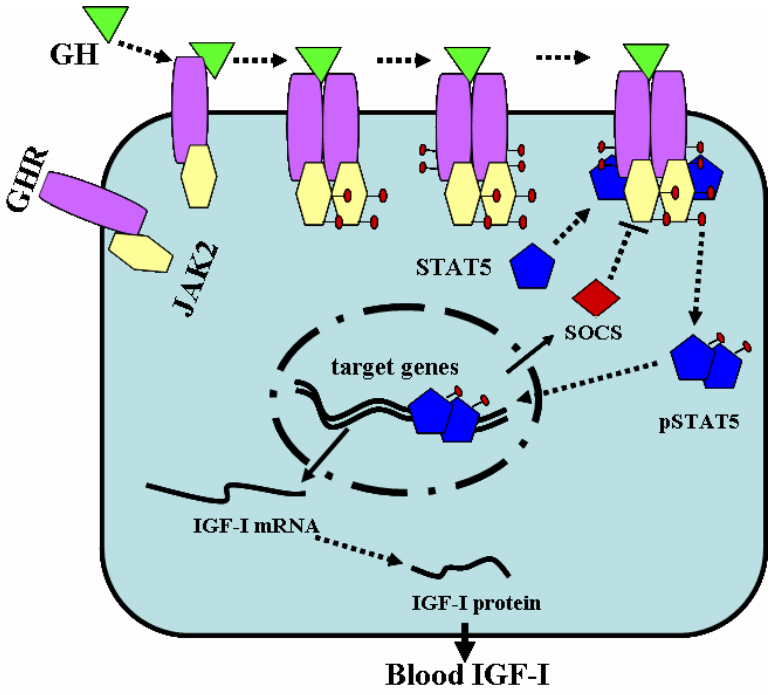


Figure 1-1. GH regulation of gene expression.

In the rest status, JAK2 is associated with the intracellular domain of membrane GHR. The binding of GH to GHR induces dimerization and conformational changes in the JAK2-associated receptors, bringing two JAK2 molecules to a closer juxtaposition so that the JH1 tyrosine residues on one JAK2 are rapidly phosphorylated and activated by another JAK2, reciprocally (Lindauer et al., 2001). Subsequently, the activated pJAK2 phosphorylates JAK2-associated receptors, which provide recruitment and docking sites for cytoplasmic transcription factor STAT5 protein. The JAK2 then phosphorylates STAT5 when they associate with receptors. Activated pSTAT5 then dissociates from the receptor complex, forms active dimers via the SH2 domain, translocates to the nucleus to bind specific DNA sequences, and induces the transcription of the target genes, including IGF-I and SOCS (O'Shea et al., 2002). SOCS proteins then inhibit GHR signaling and attenuate the expression of pSTAT5-dependent genes (Matsumoto et al., 1997; Karlsson et al., 1999; Metcalf et al., 2000; Greenhalgh and Alexander, 2004).

Nutritional regulation of circulating IGF-I

Appropriate intake of nutrients is important for optimal growth and health. Nutritional deprivation causes growth arrest and loss of cell mass. On the other hand, increased nutritional intake promotes growth rate and increases serum IGF-I level, and there is a highly positive correlation between weight gain and serum IGF-I concentration (Nam et al., 1990; Hannon et al., 1991; Stick et al., 1998; Thorn et al., 2006). Given its growth-stimulating and anabolic effects, the changes in IGF-I likely mediate the changes in growth and metabolism under different nutritional status.

Effect of food deprivation and refeeding on circulating IGF-I concentrations

Food deprivation triggers both instant and long-term metabolic responses in animals. During food deprivation, energy is first provided by hepatic glycogenolysis and glycogen, subsequently by lipid mobilization from adipose tissue, and finally from muscle proteolysis (Zhang et al., 2001a; Wang et al., 2006). The IGF-I is an important anabolic hormone that regulates cellular metabolism and somatic growth. As discussed previously, IGF-I stimulates glucose uptake and glycogen synthesis, increases amino acid uptake and protein synthesis, decreases proteolysis and nitrogen excretion, and inhibits lipolysis (Schoenle et al., 1982; Boulware et al., 1992; Fryburg, 1994; Giordano et al., 1995). Therefore, it is not surprising that IGF-I is low in blood during food deprivation (Ho et al., 1988; Bass et al., 1991; Kirby et al., 1993; Wang et al., 2003).

After food deprivation, adequate energy and protein are essential for restoration of circulating IGF-I concentration. Refeeding for 5 d with an adequate energy and protein diet restored serum IGF-I to ~70% of its level prior to food deprivation. However, a protein-deficient (32% of control) isocaloric diet resulted in a ~50% restoration, and energy deficiency led to a

further decrease in serum IGF-I compared to food deprivation (Isley et al., 1983), indicating that energy may be more critical for restoration of IGF-I after food deprivation. Further studies indicated that an energy threshold is required for dietary protein intake to restore circulating IGF-I after food deprivation (Isley et al., 1984).

Effect of feeding level on circulating IGF-I concentrations

Long-term food restriction has a suppressing effect on serum IGF-I (Oster et al., 1995; Fenwick et al., 2006), whereas over-feeding increases serum IGF-I concentration in steers (Elsasser et al., 1989). However, moderate food restriction (from 80% to 100% of ad libitum) did not affect serum IGF-I concentration as long as daily intakes of protein, vitamins and minerals were not reduced (Stick et al., 1998), again suggesting there is a threshold of energy level for nutritional intake to affect circulating IGF-I concentration.

The response of serum IGF-I to exogenous GH administration is also affected by feeding level. Steers that experienced a negative nitrogen status due to underfeeding had an attenuated IGF-I response to bGH compared to well-fed animals (Elsasser et al., 1989).

Effect of dietary energy on circulating IGF-I concentrations

There are consistent reports that serum IGF-I levels were reduced by dietary energy restriction (Straus and Takemoto, 1991; Smith et al., 1995; Kritsch et al., 2005). Plasma IGF-I was restored to normal level when enough dietary energy was re-supplied (Zhu et al., 2005).

Different sources of dietary energy also differ in regulation of circulating IGF-I. Despite isocalorie and iso-protein, the diet providing carbohydrate as primary energy source had more potent effect to increase serum IGF-I response to GH than the diet using lipid as primary energy source (Snyder et al., 1989).

Effect of dietary protein on circulating IGF-I concentrations

Animals fed higher quantity or quality of protein grow faster and show higher plasma IGF-I concentrations compared to animals fed reduced or no dietary protein (Elsasser et al., 1989; Takahashi et al., 1990; Miura et al., 1992; Smith et al., 1995; Lee et al., 2005). When rats were fed a diet without protein for 1 wk, plasma immunoreactive and total IGF-I, which were found in the 40 kDa and 150 kDa complex respectively, were significantly lower than in the rats fed a diet containing wheat gluten or a diet with casein (isonitrogenous with wheat gluten diet) (Takahashi et al., 1990). Circulating IGF-I was lower when animal experienced a negative nitrogen balance (Smith et al., 1995). Therefore, the quantity of dietary protein affects circulating IGF-I concentration.

The effect of dietary protein quality on circulating IGF-I seems not as significant as dietary protein quantity when they contain the same amount of dietary crude protein. Rats fed a diet with casein had more plasma immunoreactive IGF-I, but plasma total IGF-I was not different from the wheat gluten diet or diet with maize-gluten meal, both of which are lower in lysine and some other essential amino acids compared to casein. Additional amino acids such as lysine, threonine or tryptophan to the wheat gluten or maize-gluten meal diet did not increase circulating IGF-I (Takahashi et al., 1990).

The effect of dietary protein on circulating IGF-I is also affected by available dietary energy level. Although the diet with lower protein (CP: 8%) and metabolic energy (ME: 1.96 Mcal /kg dry matter) decreased plasma IGF-I in beef cattle, increasing dietary protein from 11% to 14% did not affect plasma IGF-I level. However, when dietary ME was 2.67 Mcal ME/kg dry matter, plasma IGF-I concentration was increased in parallel to increased CP (Elsasser et al., 1989).

Effect of other nutrients on circulating IGF-I concentrations

Some minerals and vitamins have been reported to alter circulating IGF-I concentrations. Magnesium (Mg)-deficiency for 12 d reduced serum IGF-I by 44%, and by 60% after 3 weeks of deficiency. Mg repletion restored serum IGF-I after 2 weeks of treatment (Dorup et al., 1991). Higher dietary Ca was found to restore both body weight and plasma IGF-I level in Vitamin D receptor (VDR) knockout mice (Song et al., 2003). Vitamin A (VA)-deficient rats due to dietary VA deficiency had a lower body weight and lower serum IGF-I concentration (Fu et al., 2002).

Nutritional regulation of liver IGF-I gene expression

IGF-I mRNA content in the liver responds to dietary nutrients and feeding level. There was a significant decrease in liver IGF-I mRNA level when animals experienced negative energy balance due to dietary energy restriction (Straus and Takemoto, 1991; Kritsch et al., 2005; Fenwick et al., 2006). An increase in liver IGF-I mRNA abundance was achieved in animals with increased dietary protein (Takahashi et al., 1990; Miura et al., 1992; Lee et al., 2005). Vitamin A-deficient rats due to dietary vitamin A deficiency expressed 40% less liver IGF-I mRNA abundance (Fu et al., 2002). Long-term malnutrition suppressed liver IGF-I mRNA expression (Oster et al., 1995; Fenwick et al., 2006).

The changes in liver IGF-I mRNA in response to changed nutritional intake may be due to the change in transcriptional rate or mRNA stability or both. Nuclear run-on assay indicated that the change in liver IGF-I mRNA abundance in response to dietary protein change is primarily due to changed mRNA stability, and the difference in IGF-I transcriptional rate is only 30% between protein-free diet and casein diet (Noguchi, 2000).

Nutritional regulation of serum GH and liver GHR

In short-term food deprivation, serum GH concentration is increased, and the increase is due to increased GH pulse frequency and pulse amplitude (Ho et al., 1988; Fairhall et al., 1990; Maccario et al., 2000; Kosior-Korzecka et al., 2006). However, a reduction of GH binding was found in the liver of food-deprived rats (Villares et al., 1994), cattle (Breier et al., 1988) and sheep (Bass et al., 1991). This reduction is associated with decreased serum GH-binding protein (GHBP) and liver GHR mRNA abundance (Villares et al., 1994; Fenwick et al., 2006), indicating that GHR gene expression is impaired during food restriction or deprivation. Therefore, despite the increase in GH secretion, the action of GH is diminished due to decreased GHR and/or its post-signaling pathway under food deprivation.

Besides food deprivation, changes in the levels of some nutrients have also been reported to affect serum GH concentration. Injection of glucose to fasted guinea pigs blunted the elevated blood GH levels during food deprivation (Fairhall et al., 1990). Dietary zinc and magnesium deficiency decreases GH release (Nishi et al., 1989; Dorup et al., 1991). Zinc can rapidly stimulate ^{125}I-hGH specific binding to isolated rat adipocytes (Cunningham et al., 1990). Addition of amino acid, such as lysine, arginine, ornithine or glutamine can increase pituitary GH secretion (Cree et al., 1985; Bennek et al., 1999). Increasing dietary protein decreases blood GH levels, and this decrease may be due to the decreased GH pulse amplitude (Clarke et al., 1993).

Role of insulin in nutritional regulation of circulating IGF-I

Insulin is a 51-amino acid anabolic hormone that primarily regulates carbohydrate metabolism (Derewenda et al., 1989). Serum insulin is highly related to nutritional status. Food deprivation and malnutrition markedly decrease pancreatic insulin content and secretion, and

plasma insulin concentrations (Tanigawa et al., 1989; Holness, 1996; Oster et al., 1996; Louveau et al., 2000; Kosior-Korzecka et al., 2006), while a meal or glucose administration increases insulin secretion (Tanigawa et al., 1989).

In streptozotocin-induced diabetic rats, insulin treatment restored serum IGF-I and increased the amount of GH binding sites in the liver (Baxter et al., 1980). In human hepatoma cell line HuH7, insulin increased both mRNA and total protein of GHR, resulting in an increase in GH binding (Leung et al., 2000). Insulin also affects GH-induced post-receptor signaling. Short-term insulin treatment of liver-derived cells selectively activated GH-induced MEK/ERK phosphorylation independent of JAK2, Ras and Raf-1, and it was likely resulted from increased cell membrane translocation of MEK1/2 (Xu et al., 2006). Insulin can induce tyrosine phosphorylation of JAK2 and STAT5 in some cell types, such as Kym-1 rhabdomyosarcoma cells (Storz et al., 1999). These results suggest that circulating IGF-I response to nutritional change may be mediated by insulin through regulating GHR and post-receptor signaling pathway.

Role of leptin in nutritional regulation of circulating IGF-I

Leptin is expressed in adipose tissue and involved in the regulation of food intake, body weight and whole body energy balance (Frederich et al., 1995; Wang et al., 1997; Barb et al., 1998). Leptin has been shown to reduce body fat in vivo (Wang et al., 1997), and circulating leptin rises continuously with increasing adiposity (Ostlund et al., 1996). Serum leptin is higher in well-fed animals than food-restricted animals (Kosior-Korzecka et al., 2006; Thorn et al., 2006).

Leptin receptors are predominantly expressed in the hypothalamic regions (Lin et al., 2000). Leptin also stimulates GH secretion (Carro et al., 1997; Barb et al., 1998). Serum leptin

was found positively correlated with serum IGF-I concentration (Park et al., 2001; Petridou et al., 2005). Administration of leptin increased liver IGF-I mRNA abundance, although it did not affect the relative abundance of IGF-I mRNA in skeletal muscle and adipose tissue (Ajuwon et al., 2003). These results together suggested that leptin may mediate the circulating IGF-I response to nutritional changes.

Role of Vitamin D in nutritional regulation of circulating IGF-I

Vitamin D and Vitamin D receptor (VDR) are involved in IGF-I gene expression. A positive correlation between serum free IGF-I and 25-(OH) vitamin D3 was found in man without 25-(OH) D3 deficiency (Gomez et al., 2004). The IGF-I expression was down-regulated in VDR knockout mice (Song et al., 2003). Vitamin D analog paricalcitol was found to up-regulate the expression of IGF-I in human coronary artery smooth muscle cells (Wu-Wong et al., 2006). It is known that the binding of 1,25-dihydroxyvitamin D3 or its analogs to VDR activates VDR, interacts with retinoid X receptor (RXR), and then recruits cofactors to form the VDR/RXR/cofactor complex, which then binds to Vitamin D response elements in the promoter region of target genes to regulate gene transcription (Polly et al., 1997). However, the mechanism for Vitamin D regulation of IGF-I gene expression is unclear.

Nutritional regulation of serum IGFBP-3

The binary complexes of IGF-I/IGFBP-3 function as vehicle for IGF-I to be delivered to the extravascular tissues, whereas the ternary complexes of IGF-I/IGFBP-3/ALS function as a reservoir for circulating IGF-I and decrease the clearance of IGF-I from blood (Binoux and Hossenlopp, 1988; Yakar et al., 2002b). Therefore, the change in serum IGFBP-3 or ALS or both will modulate the concentrations of circulating IGF-I response to nutritional change.

Both diertay energy and protein can affect serum IGFBP-3 level. In young adult rats, 30 days of 60% caloric restriction decreased serum IGFBP-3 level, whereas 40% caloric restriction had no impact on serum IGFBP-3 (Oster et al., 1995). Dietary protein restriction decreased serum IGFBP-3 (Oster et al., 1995; Smith et al., 1995; Takenaka et al., 1996; Higashi et al., 1998). However, increasing dietary protein did not alter plasma IGFBP-3 level, although plasma IGF-I was increased (Clarke et al., 1993).

The changes in blood IGFBP-3 in response to nutritional changes may be due to changes in hepatic IGFBP-3 gene expression, clearance from the bloodstream and/or degradation in blood. In cows experiencing severe negative energy balance, hepatic IGFBP-3 mRNA abundance was reduced (Fenwick et al., 2006). Under dietary protein deficiency, liver IGFBP-3 mRNA abundance is also decreased (Higashi et al., 1998). However, there are some reports showing that liver IGFBP-3 mRNA abundance and transcription rate did not change although plasma IGFBP-3 concentration was decreased during dietary protein malnutrition (Takenaka et al., 1996). The IGFBP-3 is secreted as a phosphoprotein (Hoeck and Mukku, 1994; Coverley and Baxter, 1997). Dephosphorylation does not alter IGFBP-3 binding ability to IGF-I (Hoeck and Mukku, 1994; Coverley et al., 2000), but phospho-IGFBP-3 showed a lower affinity for ALS and poor association with cell surfaces (Coverley et al., 2000). Phospho-IGFBP-3 is more stable and is more resistant to proteolysis (Coverley et al., 2000). Proteolysis of IGFBP-3 is commonly found in pregnancy and catabolic conditions (Wu et al., 1999). Growth hormone deficiency may induce IGFBP-3 proteolysis, since sera from hypophysectomized rats or dwarf mice had strong IGFBP-3 proteolytic activity (Koedam et al., 1998). Proteolysis of IGFBP-3 lowers its affinity for IGF-I and leads to dissociation of the ternary complex (Lee and Rechler, 1996). Serum IGFBP-3 proteolysis can be inhibited completely by EDTA, and the inhibition can be reversed

by zinc ions (Lee and Rechler, 1996; Koedam et al., 1998; Wu et al., 1999), indicating that the protease is cation-dependent.

Nutritional regulation of serum ALS

ALS is critical for maintaining circulating IGF-I and IGFBP-3 by sequestering them into a stable ternary complex, inhibiting IGF-I transendothelial transport and decreasing clearance of IGF-I from the circulation (Payet et al., 2004). Food deprivation decreases circulating ALS level (Dai and Baxter, 1994; Frystyk et al., 1999; Kong et al., 2002). Malnutrition during late pregnancy due to placental insufficiency reduced ALS gene expression in the liver of lamb fetuses (Rhoads et al., 2000a). In early postnatal life, lower nutritional intake also decreased hepatic ALS expression in lamb (Rhoads et al., 2000a). Caloric restriction reduced serum ALS in rats (Oster et al., 1996).

Circulating ALS is mainly from liver, in which hepatocyte is major cell type for synthesis of ALS (Chin et al., 1994; Scharf et al., 1995; Delhanty and Baxter, 1996). Liver ALS gene expression is induced during the late fetal stage, dramatically increased in the first few weeks after birth, and changed little thereafter (Nimura et al., 2000; Rhoads et al., 2000b; Lee et al., 2001). Food deprivation decreases liver ALS gene expression and serum ALS concentration (Frystyk et al., 1999; Kong et al., 2002). In the cows with severe negative energy balance, hepatic ALS mRNA was reduced (Fenwick et al., 2006). However, some conflicted reports indicated that liver ALS mRNA content was not affected by protein deprivation for 1 week (Takenaka 1996), and that fasting for 24 or 48 h did not decrease hepatic ALS mRNA abundance despite decreased serum ALS (Dai and Baxter, 1994).

Nutritional regulation of ALS may be mediated by cyclic adenosine 3',5'-monophosphate (cAMP) and changes in circulating GH levels or tissue GH sensitivity. Increasing intracellular

cAMP in primary hepatocytes attenuates liver ALS mRNA levels or protein secretion dependent on GH status (Delhanty and Baxter, 1998; Scharf et al., 2001). In the presence of GH, both mRNA levels and protein secretion are suppressed by cAMP. However, under basal conditions cAMP mainly suppressed ALS secretion. cAMP may affect ALS gene transcription through blunting the GH signaling pathway (Delhanty and Baxter, 1998).

ALS is a glycoprotein containing about 20 kDa of carbohydrate. There are three potential N-linked oligosaccharide sites in the leucine-rich repeat (LRR) domain (Janosi et al., 1999b). Complete deglycosylation was found to decrease the ability of ALS to associate with IGFBP-3 (Janosi et al., 1999a).

Summary

IGF-I is an important regulator of anabolic metabolism and somatic growth. Circulating IGF-I primarily comes from liver, in which IGF-I gene expression is regulated by GH and nutritional intake. The IGFBP-3 and ALS, which are mainly secreted from liver and GH-dependent, are involved in regulating the availability of circulating IGF-I for target tissues and the half-life of IGF-I in the bloodstream. However, the molecular mechanism underlying nutritional regulation of circulating IGF-I availability is largely unknown. For example, does nutritional regulation of liver IGF-I, IGFBP-3 and ALS gene expression occur at the transcriptional or translational level or both? Is degradation of IGF-I in serum altered under different feeding levels? This dissertation research was designed and conducted to address these and other related questions.

Chapter II

Effects of feeding levels on serum insulin-like growth factor-I response to growth hormone in cows

ABSTRACT

Growth hormone (GH) and insulin-like growth factor-I (IGF-I) are two polypeptide hormones with anabolic effects on somatic growth and metabolism. Serum IGF-I is mainly derived from liver, and serum IGF-I level and liver IGF-I gene expression are responsive to many factors, including nutrition and growth hormone. This research was conducted to study the effects of feeding levels on circulating IGF-I response to GH administration in cows and the underlying mechanism. Twenty non-pregnant and non-lactating cows were randomly assigned to two diet sequences. One group of cows had *ad libitum* access to tall fescue hay from day 0 (d0) to d98. The other group of cows had *ad libitum* access to hay from d0 to d7, had corn-based concentrates in addition to free access to hay from d8 to d63, and was moderately food-restricted from d64 to d98. On d0, d56 and d91, each cow was injected subcutaneously with 500 mg of prolonged-release bovine GH. Blood samples were collected each day during the week of GH administration, and subsequently once per week between two GH administrations. Liver biopsies were performed on the day before and the 7^{th} day after GH administration (i.e. on d0, d7, d56, d63, d91 and d98). Supplementation of concentrates enhanced average daily gain response to GH administration, whereas food restriction blunted the response. Supplementation of concentrates or food restriction did not change serum basal IGF-I, IGFBP-3 or ALS levels, or their mRNA abundance in the liver. Growth hormone increased serum IGF-I concentration and this increase was greater in the cows supplemented with concentrates than in the cows fed hay only. This

greater serum IGF-I response to GH was not due to a lesser degradation of IGF-I or greater GH concentration in serum. Although it did not affect GH-stimulated IGF-I total mRNA expression in the liver, supplementation of concentrates was associated with a greater increase in the amount of GH-stimulated liver IGF-I mRNA bound to polysomes, suggesting that increased nutritional intake and GH together increased the translation of IGF-I mRNA in the liver. Supplementation of concentrates did not affect basal or GH-stimulated IGFBP-3 or ALS mRNA expression in the liver, but it enhanced GH-stimulated increases in serum IGFBP-3 and ALS abundances. These greater increases may be due to a lesser clearance of IGFBP-3 and ALS from the bloodstream as a result of increased serum IGF-I and hence increased formation of IGF-I / IGFBP-3 / ALS complexes. In conclusion, increased nutritional intake might increase translation of GH-stimulated IGF-I mRNA in the liver, and this increase might contribute to a greater increase in serum IGF-I and hence a greater retention of both IGFBP-3 and ALS in the bloodstream.

Key Words: Nutrition, Insulin-like growth factor-I, Growth hormone, IGF binding protein-3, Acid-labile subunit, Cattle

INTRODUCTION

Growth hormone, also called somatotropin (ST), is a 191-amino acid polypeptide hormone synthesized in the anterior pituitary gland (Roux et al., 1982). Growth hormone has growth-promoting and anabolic effects (Van Vliet et al., 1987; Welbourne et al., 1989; Zhu et al., 1997). It increases milk production in dairy cows, increases meat production and reduces carcass fat content in growing pigs (Etherton et al., 1993). In 1993, a prolonged-release bovine somatotropin (bST) from Monsanto Company was approved to be used to stimulate milk production in dairy cows by the FDA, and currently more than 3 million cows in the United States are supplemented with bST (Akers, 2006).

A major biological effect of GH is to stimulate IGF-I biosynthesis, especially in liver (Mathews et al., 1986; Ramsay et al., 1995). The IGF-I is a polypeptide growth factor important for growth and metabolism. Liver-specific IGF-I gene inactivation in mice indicated that 75% of circulating IGF-I comes from liver (Sjogren et al., 1999) and that liver-derived IGF-I is important for normal carbohydrate and lipid metabolism (Sjogren et al., 2001). Circulating IGF-I concentration is also regulated by a group of IGF-I related peptides, including IGFBP-1 through IGFBP-6, and ALS. Under normal physiological conditions, most of serum IGF-I is in a binary complex of IGF-I/IGFBP-3 functioning as a vehicle for IGF-I to be delivered to the IGF-I-target tissues, or a ternary complex of IGF-I/IGFBP-3/ALS serving as a reservoir for circulating IGF-I (Binoux and Hossenlopp, 1988; Albiston and Herington, 1990; Yakar et al., 2002b).

The effects of GH in animals depend on the nutritional status of the animals. Overfeeding of either dietary energy or crude protein boosted blood IGF-I response to GH administration (McGuire et al., 1992), while food restriction or deprivation decreased blood IGF-I response to GH (Breier et al., 1988; Villares et al., 1994). The mechanism by which nutrition regulates blood

IGF-I response to GH is still not well understood. This study was conducted to explore this mechanism. Specifically, we determined whether serum IGF-I degradation, serum IGFBP-3 and ALS concentrations, GH-stimulated expression of liver IGF-I, IGFBP-3 and ALS mRNAs and proteins were affected by feeding levels in cattle.

MATERIALS AND METHODS

Animals, treatments and sample collection

Twenty non-pregnant and non-lactating cows were randomly assigned to two side-by-side pens, 10 cows a pen. The age or initial body weight was not different between the two pens of animals: 4.4 ± 0.4 years old versus 4.6 ± 0.4 years old (P = 0.72) and 541.0 ± 9.5 kg versus 538.7 ± 9.5 kg (P = 0.87). The animal protocol was approved by the Virginia Tech Animal Care Committee.

One pen of cows had *ad libitum* access to tall fescue hay (*Festuca arundinacea*) from the first day of the experiment, designated day 0 (d0), to the last day of the experiment (d98), and these cows were named the HAY group cows. The cows in the other pen, which were named the CORN group cows, had *ad libitum* access to tall fescue hay from d0 to d7, had 9.1 kg of concentrates (91.2% corn, 8% soybean meal and 0.8% urea) per cow each day in addition to *ad libitum* access to hay from d8 to d63, and had restricted access to hay from d64 to d98 (Figure 2-1). The CORN group cows consumed 90% of hay that was consumed by the HAY group cows from d64 to d98. All cows had *ad libitum* access to mineral and vitamin mixtures containing calcium (8.50%-10.20%), phosphorus (>7.90%), salt (17.00%-20.00%), magnesium (>12.00%), potassium (>1.00%), sulfur (>0.50%), cobalt (>50 ppm), copper (>300 ppm), iodine (>60 ppm), manganese (>1200 ppm), selenium (60 ppm), zinc (>1300 ppm), vitamin A (200.46 IU/g), vitamin D3 (55.11 IU/g) and vitamin E (0.55 IU/g). On d0, d56 and d91, each cow was injected subcutaneously with 500 mg of recombinant bovine growth hormone in a prolonged-release formula (Monsanto Company, St. Louis, Missouri). Body weight of each cow was recorded once a week at 0900.

A blood sample was collected from each cow from the jugular vein using a 9-mL vacuum tube containing serum clot activator (Greiner bio-one VACUETTE America, Monroe, North Carolina) immediately before GH administration and also every 24 hours following GH administration for 7 consecutive days. Blood samples continued to be collected from each cow once a week between the two GH administrations. Blood samples were allowed to clot at room temperature for 1 h, and then centrifuged at 1,800 × g at 4 °C for 25 min to separate sera, which were stored at –20 °C for future assays.

Liver biopsies were performed immediately before and the 7th day after each GH administration (i.e., on d0, 7, 56, 63, 91 and 98) as described previously (Oxender et al., 1971). Liver samples were immediately frozen in liquid nitrogen and stored at –80 °C.

Due to limited amount of tissue, the liver samples of the 10 animals from each pen were randomly divided between assays, with five animals being used for each assay. The two groups of five animals from each pen were not different in age and body weight on d0. The two groups of HAY group cows weighed 538.5 ± 13.0 kg and 543.5 ±13.0 kg on d0 (P = 0.79), and were 4.2 ± 0.5 and 4.6 ± 0.5 years old (P = 0.62). The two groups of CORN group cows weighed 538.1 ± 15.4 kg and 539.4 ± 15.4 kg (P = 0.95), and were 5.2 ± 0.5 and 4.0 ± 0.5 years old (P = 0.14).

Radioimmunoassay (RIA)

Serum concentrations of total IGF-I were determined by radioimmunoassay following an acid-ethanol cryoprecipitation extraction to remove IGF binding proteins (Purup et al., 1993). One hundred microliters of serum sample were mixed with 900 μL extraction solution (87.5% ethanol and 12.5% 2 N HCl). After 1 h of incubation at room temperature, the mixed solution was centrifuged at 13,000 × g for 10 min at 4 °C, and 500 μL of the supernatant

were neutralized by 200 μL of 0.855 *M* Tris base. After 1 h of incubation at – 20 °C, the neutralized solution was centrifuged at 3,000 × g for 30 min, and 30 μL of supernatant were used for each RIA. One hundred microliters of ^{125}I-labeled IGF-I (2.77×10^4 dpm) and 100 μL of the mouse antiserum (a gift of Dr. Bernard Laarveld, University of Saskatchewan) raised against bovine IGF-I were added to the 30 μL extracted solution. After incubation for 24 h, 100 μL of the goat-anti-mouse second antiserum (Sigma Chemical Company, St. Louis, MO, USA) were added to each tube. After incubation at 4 °C for 72 h, 1 mL of phosphate-buffered saline (PBS) was added to each tube, and the tube was then centrifuged at 1,560 × g for 30 min at 4 °C. The supernatant were decanted and the radioactivity of the pellet that remained in the tube was measured by gamma counting. The detection range of the RIA was 23.3 to 2,986.7 ng/mL.

Serum concentrations of growth hormone were also determined by radioimmunoassay (Akers et al., 1986). Three hundred microliters of serum sample or GH standard (recombined bovine GH, rbGH) were added to RIA buffer to a final volume of 500 μL. Subsequently, 100 μL of ^{125}I-labeled rbGH and 100 μL of rabbit anti-ovine GH (at 1: 70,000 dilution) were added to each tube. After 24-h incubation at room temperature, 100 μL of secondary antibody (at 1: 10 dilution) were added. The tubes were incubated further for 72 h at 4 °C. One and a half milliliters of PBS were then added to each tube followed by centrifugation at 1,500 × g for 30 min. The supernatant were decanted and the radioactivity of the pellet that remained in the tube was measured by gamma counting. The detection range of this RIA was 0.17-42.62 ng/mL.

Stability assay of ^{125}I-labeled IGF-I in serum

An *in vitro* method was developed to test the stability of ^{125}I-labeled IGF-I in serum. Five microliters of ^{125}I-labeled IGF-I (1.52×10^5 dpm) were incubated at 37 °C for 12 h in 50 μL of serum. Fifty microliters of H_2O and 50 μL of 10 μg/μL proteinase K (Fisher Scientific, Fair Lawn, NJ) were used as negative and positive controls, respectively. Following the incubation, 5.5 μL of the solution were resolved by electrophoresis on 8% SDS-polyacrylamide gels at 150 volt for 1.5 h. After electrophoresis, the gels were dried for 1 h on a Model 583 gel dryer (Bio-Rad Laboratories, Hercules, CA) and exposed to phosphor screens. The exposed phosphor screens were scanned on a Molecular Imager FX System (Bio-Rad Laboratories, Hercules, CA). The densities of undegraded ^{125}I-IGF-I were quantified by using the ImageJ program (http://rsb.info.nih.gov/ij/index.html).

RNA extraction and quantification

Total RNA from liver samples was isolated using TRI Reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. Briefly, frozen tissue (about 0.5 g) was homogenized in 5 mL of TRI Reagent at 20,000 rpm for 45 sec using a Virtis homogenizer model 225318 (Virtis, Gardiner, NY). One milliliter of chloroform was added to the homogenates, and then 2.5 mL of isopropanol were added to the collected aqueous supernatant. Total RNA was collected by centrifugation at 12,000 × g for 10 min at 4 °C, and redissolved in diethypyrocarbonate (DEPC)-treated water. RNA concentrations were measured based on the absorbance at 260 nm in a Spectrophotometer U-2810 (Digilab Hitachi, San Jose, CA), and RNA quality was verified by electrophoresis on 1% agarose gels containing 20% of formaldehyde (v/v).

Ribonuclease protection assay (RPA)

The plasmid for synthesis of total IGF-I mRNA detection probe was a pGEM-T Easy plasmid containing a 200-bp cDNA insert that corresponded to 137 bp of exon 3 and 63 bp of exon 4 of the bovine IGF-I gene (Kobayashi et al., 1999). The plasmids used to synthesize the bovine IGFBP-3 and ALS mRNA probes were pGEM-T Easy-based plasmids containing a 232-bp bovine IGFBP-3 and a 197-bp bovine ALS cDNA inserts, respectively (Jiang, unpublished data). The plasmid for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a pGEM-T Easy plasmid containing a 90-bp bovine GAPDH cDNA (Wang et al., 2003).

The radioactive antisense probes for RPA were synthesized by using α-^{32}P-CTP and the Riboprobe Combination Systems kit (Promega, Madison, WI). Briefly, in vitro transcription was performed in a 25 μL of solution (5 μL of 5 × transcription buffer, 2.5 μL of 0.1 *M* DTT, 1 μL of 10 m*M* ATP, 1 μL of 10 m*M* GTP, 1 μL of 10 m*M* UTP, 1 μL of 100 μM CTP, 1 μL of RNase inhibitor, 0.5 μg of linearized DNA template, 5 μL of α-^{32}P-CTP and 1 μL of RNA polymerase), and incubated at 37 °C for 1 h. Following the incubation, 2 μL of RNase-free DNase I were added to the solution, and then the solution was incubated at 37 °C for 20 min. Following the digestion, free α-^{32}P-CTP was removed by filtrating the solution through Quick Spin Sephadex G-50 columns (Roche Molecular Biochemicals, Indianapolis, IN). The radioactivity of the purified probe was determined by liquid scintillation counting in a Backman LS 5000 TA (Beckman Instruments Inc., Fullerton, CA).

The RPA was carried out by using the RPA II kit (Ambion, Austin, TX). Twenty micrograms of total RNA were used in the RPA of liver IGF-I, IGFBP-3 and ALS mRNA. There were two hybridization reactions: one contained 1.05×10^6 dpm of probe for IGF-I

mRNA and ~4.5 × 10^5 dpm of probe for GAPDH mRNA, and the other had 8.0 × 10^5 dpm for ALS mRNA, 5.0 × 10^5 dpm for IGFBP-3 mRNA and ~4.5 × 10^5 dpm of probe for GAPDH mRNA. The hybridization was incubated at 42 °C for 16 h. Following the hybridization, non-hybridized RNA fragments were digested by RNase A/T1 mixture at 37 °C for 40 min. After digestion, the ribonuclease-protected RNA fragments were resolved by electrophoresis on 6% acrylamide gels containing 7 *M* urea. After gel electrophoresis, the radioactivity of the ribonuclease-protected bands was detected by exposure to phosphor screens and quantified by using the ImageJ program as described above. The density of the protected IGF-I, IGFBP-3 or ALS mRNA band was normalized to that of the protected GAPDH mRNA band in the same RNA sample, and the normalized density was used to represent the abundance of the corresponding mRNA.

Preparation of liver whole cell lysates

For extraction of liver whole cell lysates, 0.5 g of frozen liver tissue was homogenized in 2 mL of ice-cold lysis buffer A (10 m*M* Tris, 1 m*M* EGTA, 1 m*M* EDTA, 150 m*M* NaCl, and 1% Triton X-100, pH 7.6) containing protease inhibitors (1 m*M* Na_3VO_4, 1 m*M* Na pyrophosphate, 10 m*M* NaF, 1 m*M* PMSF, 10 mg/L aprotinin and 10 mg/L Leupeptin) at 20,000 rpm for 1 min. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C, and the supernatant were centrifuged again at 12,000 × g for 10 min at 4 °C. The final supernatant were collected and stored at –80 °C.

Western blot analysis

Liver whole cell lysates (40 μg of protein) were resolved by electrophoresis on 10% SDS-polyacrylamide gels, and then electro-transferred to nitrocellulose membranes. After being blocked with 5% milk in TBST (Tris buffered saline contained 0.1% Tween-20) for 1 h

at room temperature, the nitrocellulose membrane was incubated with a primary antibody at 4 °C overnight. The primary antibody for detection of ALS was anti-ALS 1082 antibody at the dilution of 1:3000, a rabbit antiserum raised against a KLH-conjugated peptide with 28 residues of bovine ALS mapping at the carboxyl-terminal (Kim et al., 2006). The endogenous control β-actin was detected by an antibody from Cell Signaling Technology (Danvers, MA) at the dilution of 1:500. After being washed with TBST for three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit IgG-HRP antibody, Santa Cruz biotechnology, Santa Cruz, CA) at 1: 5000 dilution for 2 h at room temperature. After three times of washing with TBST, the membrane was incubated in SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescence signal was detected with X-ray films, and quantified using the ImageJ program as described above.

For Western blot analysis of serum ALS, 0.5 μL of serum was used and anti-ALS 1082 antibody was used at 1:5000 dilution. The remaining steps were as same as the Western blot analysis for liver ALS protein.

Western ligand blot

This assay was used to quantify serum IGFBP-3.. Four microliters of serum were heated at 60 °C for 10 min in non-reducing Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), and then resolved by 12% SDS-polyacrylamide gels at 150 volt for 3 h. The proteins in gels were electro-transferred to nitrocellulose membranes by running at 55 V for 2 h. The membrane was washed with 3% igepal CA-630 in Tris buffered saline (TBS, pH 7.4) for 30 min, and blocked with 1% BSA in TBS (pH 7.4) for 2 h. After being washed with 0.1% Tween-20 in TBS (pH 7.4) for three times, the membrane was incubated with 1.25 ×

10^6 dpm of ^{125}I-labeled IGF-I/mL at 4 °C overnight. After being washed with 0.1% Tween-20 in TBS (pH 7.4) for three times and TBS (pH 7.4) for two times, the membrane was dried at 37 °C, and then exposed to X-ray film for 4 d at –80 °C. The density of the bands in the image was quantified by using the ImageJ program as described above.

Isolation of polysomal RNAs

Bovine liver polysomes were isolated as described for rat liver polysome isolation (Foyt et al., 1992), with minor modifications. Frozen liver tissue (approximately 0.3 g) was homogenized in 5 mL of 0.25 *M* sucrose buffer containing 200 m*M* Tris (pH 8.5), 50 m*M* KCl, 40 m*M* $MgCl_2$, 25 m*M* EGTA, 0.25 *M* sucrose, 5 m*M* dithiothreitol, 0.5 U/μL porcine Optizyme RNase Inhibitor (Fisher Scientific, Fair Lawn, NJ), and 2 μg/mL cycloheximide at 7,300 rpm for 30 sec using a Virtis homogenizer model 225318. Triton X-l00 was added to a concentration of 0.5%, and the homogenate was centrifuged at 1,100 × g for 10 min. The supernatant were collected, and Triton X-100 was added to a final concentration of 2.5%. The supernatant were incubated at 4 °C for 10 min, and then centrifuged at 12,100 × g for 15 min. The supernatant (approximately 4.5 mL) was collected again, and carefully layered on a gradient sucrose cushion with a lower layer of 4 mL of 1.9 *M* sucrose and an upper layer of 4 mL of 1.0 *M* sucrose, both in the buffer containing 200 m*M* Tris (pH 8.5), 50 m*M* KCl, 40 m*M* $MgCl_2$, 25 m*M* EGTA, 5 m*M* dithiothreitol, 0.1 U/μL porcine Optizyme RNase Inhibitor, and 2 μg/mL cycloheximide. The gradients were centrifuged at 184,000 × g for 2 h (Beckmen LE-80 ultracentrifuge, rotor 55-2Ti). The polysomal pellets were re-suspended with 1 mL of Tri-reagent for polysomal RNA isolation. Polysomal RNA was isolated as previously described for total RNA isolation.

Reverse transcription and real time-PCR (RT-PCR)

Reverse transcription coupled with real time quantitative PCR was used to quantify the abundance of IGF-I, IGFBP-3, ALS and GAPDH mRNAs in liver polysomal RNA. Total RNA were reverse-transcribed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Briefly, 0.4 μg of total RNA was reverse-transcribed in 20 μL containing 110 nmol $MgCl_2$, 10 nmol of dNTPs, 8 μU of ribonuclease inhibitor, 50 pmol of random hexamers, 0.50 μL of 50 U /μL multiscribe reverse transcriptase, and 2 μL of 10 × reverse transcription buffer. The reverse transcription reaction was performed at 25 ºC for 10 min, 48 ºC for 30 min, and 95 °C for 5 min.

The PCR was set up by using a Power Syber Green PCR Master Mix kit (Applied Biosystems, Foster City, CA). The total volume of PCR reaction was 25 μL, containing 2 μL of reverse-transcribed products that corresponded to 0.02 μg of liver polysomal RNA, 12.5 μL of Syber Green PCR Master Mix, 0.5 μL of 10 μM forward primer and 0.5 μL of 10 μM reverse primer (Table 2-1). The PCR were run on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative abundance of IGF-I, IGFBP-3 or ALS mRNA on individual days was calculated using the $2^{-\Delta Ct}$ method, and the GH-stimulated increase in mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, according to the manufacturer's directions. Ct is threshold cycle representing the fractional cycle number at which the fluorescence passes the threshold. ΔCt is the normalization of IGF-I, IGFBP-3 or ALS mRNA Ct to endogenous control GAPDH mRNA Ct; that is, ΔCt = Ct for target mRNA – Ct for GAPDH mRNA. ΔΔCt = ΔCt after GH – ΔCt before GH for the same animal.

Statistical analysis

Main effects of diet sequence (DietSeq), day and diet sequence-day interaction (DietSeq*day) were tested using the PROC MIXED procedure of SAS 9.0 (SAS Institute, Inc., Cary, NC) with cow nested within diet sequence. Diet sequence was either HAY or CORN. Dependent variables were measured on various days (Appendix Table 4).

The following model was used: $Y_{ijk} = \mu + \alpha_i + \beta_{(i)j} + \gamma_k + \alpha\gamma_{ik} + E_{ijk}$

Y_{ijk} represents dependent variable corresponding with the ith diet sequence of the jth cow on the kth day. The mean is μ. The α_i is the fixed effect of the ith diet sequence (i = 0 for HAY or 1 for CORN). The $\beta_{(i)j}$ is the random effect of the jth cow within the ith diet sequence. The γ_k is the fixed effect of the kth day. The $\alpha\gamma_{ik}$ is the effect of interaction between ith diet sequence and kth day, and E_{ijk} is residual error. The expected ANOVA is shown in Appendix Table 5.

The covariance structure was used to select Spatial Power (sp(pow)) for all analyses except the dependent variable body weight gain for which Compound Symmetry (cs) was used (refer to APPENDIX I). In SAS the Spatial Power (sp(pow)) structure is applicable for the unequally spaced data which can provide a direct generalisation of the autoregressive(1) (ar(1)) structure for equally spaced data (Zimmerman and Harville, 1991). Compound Symmetry assumes equal variances and correlations between days (Jennrich and Schluchter, 1986; Everitt, 1995).

The difference between the HAY and CORN group cows on each individual day was tested by the SLICE option in the PROC MIXED procedure. The difference among d0, d56 and d91 in the HAY or the CORN group cows (to determine the effect of feeding level), the difference between before and after GH administration (i.e. d0 value versus the average of d1

to d7 values, d56 value versus the average of d57 to d63 values, and d91 value versus the average of d92 to d98 values) in the HAY or the CORN group cows (to determine the effect of GH administration), and the difference in the GH-stimulated increase (i.e. d7 value – d0 value, d63 value – d56 value, and d98 value – d91 value) between the HAY and CORN group cows (to determine the effect of feeding level on GH response) were tested by contrasts, and then adjusted for lack of orthogonality to a Bonferroni p-value for 15 pairs of contrasts. Results are presented as LS mean ± SE.

RESULTS

Effects of feeding level and GH administration on average daily gain in cows

As shown in Figure 2-2A, the HAY group cows that had tall fescue hay *ad libitum* from d0 to d98 maintained their body weights. The CORN group cows appeared to gain in body weight from d7 to d56 and lose in body weight from d63 to d98. The average daily gain was calculated for the different time periods, that is, from d0 to d7, d7 to d56, d56 to d63, d63 to d91 and from d91 to d98. The average daily gain was affected by diet sequences ($P < 0.01$), and there were an effect of time period ($P < 0.01$) and an interaction between diet sequence and time period ($P < 0.01$). As shown in Figure 2-2B, the average daily gain of the HAY group cows was not different between the two periods of d7 to d56 and d63 to d91 ($P = 0.99$). However, the average daily gain of the CORN group cows from d7 to d56 was heavier than that from d63 to d91 ($P < 0.01$). Therefore, supplementation of concentrates increased average daily gain, whereas food restriction decreased average daily gain in cows.

The average daily gain of the HAY group cows was not different among the periods of d0 to d7, d56 to d63, and d91 to d98 ($P = 0.99$, 0.99 and 0.99, respectively). It was also not different in the HAY group cows between the periods of d7 to d56 and d56 to d63, or between the periods of d63 to d91 and d91 to d98 ($P = 0.99$ and 0.99, respectively), indicating that GH administration did not alter growth of the HAY group cows. The average daily gain of the CORN group cows from d56 to d63 was greater than that from d0 to d7 and that from d91 to d98 ($P < 0.01$ and < 0.01, respectively). The average daily gain of the CORN group cows was not different between the periods of d0 to d7 and d91 to d98 ($P = 0.68$). The average daily gain of the CORN group cows from d56 to d63 was greater than that from d7 to d56 ($P < 0.01$). These results indicated that increased nutritional intake enhanced the growth response of cows to GH administration.

Effects of feeding level and GH administration on serum IGF-I concentrations

Serum IGF-I concentrations were affected by diet sequences ($P < 0.01$). There were effects of day ($P < 0.01$) and an interaction between diet sequence and day ($P < 0.01$). As shown in Figure 2-3, basal serum IGF-I concentrations in the HAY group cows were not different among d0, d56 and d91 ($P = 0.99$, 0.99 and 0.99, respectively). Basal serum IGF-I concentrations in the CORN group cows were also not different among d0, d56 and d91 ($P = 0.99$, 0.99 and 0.99, respectively). Basal serum IGF-I concentrations were not different between the HAY and CORN group cows on d0, d56 or d91 ($P = 0.47$, 0.79 and 0.87, respectively). Supplementation of concentrates did not increase basal serum IGF-I concentrations on d14, d21, d28, d35, d42 or d49 ($P = 0.79$, 0.72, 0.51, 0.73, 0.90 and 0.86, respectively), and moderate food restriction also did not change IGF-I concentrations on d70, d77 or d84 ($P = 0.36$, 0.84 and 0.87, respectively). These results indicate that increased nutritional intake or moderate food restriction did not alter basal serum IGF-I concentration in cows.

As also shown in Figure 2-3, GH administration increased serum IGF-I concentrations in the HAY group cows from d0 to d7, from d56 to d63 and from d91 to d98 ($P < 0.01$, < 0.01 and < 0.01, respectively) and also in the CORN group cows ($P < 0.01$, < 0.01 and < 0.01, respectively). Growth hormone-stimulated serum IGF-I concentrations were not different between the HAY and CORN group cows on d1, d2, d3, d4, d5, d6 or d7 ($P = 0.89$, 0.85, 0.78, 0.86, 0.32, 0.89 and 0.68, respectively). They were also not different on d92, d93, d94, d95, d96, d97 or d98 ($P = 0.68$, 0.67, 0.64, 0.73, 0.44, 0.97 and 0.54, respectively). However, supplementation of concentrates increased GH-stimulated serum IGF-I concentrations on d57, d58, d59, d60, d61, d62 and d63 ($P < 0.01$, < 0.01, < 0.01, < 0.01, < 0.01, < 0.01 and < 0.01, respectively). Growth hormone-stimulated increase in serum IGF-I concentrations was not

different between the HAY and CORN group cows from d0 to d7, or from d91 to d98 (P = 0.99 and 0.99, respectively), but it was greater in the CORN group cows than in the HAY group cows from d56 to d63 (P < 0.01). Therefore, GH administration increased serum IGF-I concentration, and increased nutritional intake further amplified this effect of GH administration.

Effects of feeding level and GH administration on serum GH concentrations

Serum GH concentrations were measured to determine whether the differences in GH-stimulated serum IGF-I between different feeding levels were due to different serum GH concentrations. Serum GH concentrations were not affected by diet sequences (P = 0.48), and there was no interaction of diet sequence with day (P = 0.93), but there was an effect of day (P < 0.01). As shown in Figure 2-4, serum GH concentrations prior to GH administration were not different among d0, d56 and d91 in the HAY group cows (P = 0.99, 0.99 and 0.99, respectively), nor were they in the CORN group cows (P = 0.99, 0.99 and 0.99, respectively).

Growth hormone administration increased serum GH concentrations in the HAY group cows from d56 to d63 and from d91 to d98 (P < 0.01 and < 0.01, respectively), but not from d0 to d7 (P = 0.12). Growth hormone administration increased serum GH concentrations in the CORN group cows from d0 to d7, from d56 to d63, and from d91 to d98 (P < 0.05, < 0.01 and < 0.01, respectively). Therefore, GH administration increased serum GH levels, but supplementation of concentrates or moderate food restriction did not alter serum GH concentrations in cows, indicating that the greater increase in GH-stimulated serum IGF-I in the CORN group cows from d57 to d63 was not due to a greater increase in serum GH concentrations.

Effects of feeding level and GH administration on IGF-I degradation in the serum

It is possible that the greater GH-stimulated increase in serum IGF-I in the CORN group cows from d57 to d63 was due to reduced IGF-I degradation in the serum. To test this possibility, the stability of ^{125}I-labeled IGF-I in sera from the two groups of cows was determined. As shown in Figure 2-5A & 2-5B, undegraded ^{125}I-IGF-I abundance was not affected by diet sequences (P = 0.99), and there was no interaction of diet sequence with day (P = 0.92). There was no difference in undegraded ^{125}I-IGF-I abundance among d0, d56 and d91 in the HAY group cows (P = 0.99, 0.99 and 0.99, respectively) or in the CORN group cows (P = 0.98, 0.99 and 0.99, respectively).

Growth hormone administration did not change ^{125}I-labeled IGF-I stability in the sera of the HAY group cows from d0 to d7, from d56 to d63, or from d91 to d98 (P = 0.99, 0.99 and 0.77, respectively), nor did it in the sera of the CORN group cows (P = 0.95, 0.99 and 0.64, respectively). These results indicate that supplementation of concentrates, moderate food restriction or GH administration did not alter IGF-I stability in the serum of cows, which suggests that the greater GH-stimulated increase in serum IGF-I in the CORN group cows from d57 to d63 was not due to lesser IGF-I degradation in the blood.

Effects of feeding level and GH administration on liver IGF-I mRNA expression

Liver IGF-I mRNA abundance was not affected by diet sequences (P = 0.98), and there was also no interaction of diet sequence with day (P = 0.11), but there was an effect of day (P < 0.01). This result was further confirmed by another RPA on the RNA samples from four additional cows from each group (APPENDIX II). As shown in Figure 2-6A & 2-6B, basal liver IGF-I mRNA abundance prior to GH administration was not different among d0, d56 and d91 in the HAY group cows (P = 0.99, 0.99 and 0.99, respectively), or in the CORN group cows (P =

0.98, 0.99 and 0.99, respectively), indicating that supplementation of concentrates or moderate food restriction did not alter basal liver IGF-I mRNA abundance in cows.

After 7 days of GH administration, liver IGF-I mRNA abundance was increased in the HAY group cows on d7 ($P < 0.01$), tended to be increased on d63 ($P = 0.054$), but was not changed on d98 ($P = 0.99$). Liver IGF-I mRNA abundance was also increased in the CORN group cows on d7 and d63 ($P < 0.05$ and < 0.01, respectively), but was not changed on d98 ($P = 0.26$). However, GH-stimulated liver IGF-I mRNA abundance was not different between the HAY and CORN group cows on d7, d63 or d98 ($P = 0.73$, 0.31 and 0.33, respectively), nor was the GH-stimulated increase in liver IGF-I mRNA between the HAY and CORN group cows from d0 to d7, from d56 to d63, or from d91 to d98 ($P = 0.99$, 0.16 and 0.92, respectively). Therefore, GH administration stimulated liver IGF-I mRNA expression. This stimulation was not affected by increased nutritional intake, but blunted by moderate food restriction.

Effects of feeding level and GH administration on liver IGF-I polysomal mRNA abundance

Liver IGF-I polysomal mRNA abundance was not affected by diet sequences ($P = 0.47$), but there were an effect of day and an interaction of diet sequence with day ($P < 0.01$ and < 0.05, respectively). Prior to GH administration, basal liver IGF-I polysomal mRNA in the CORN group cows was not different between d0 and d56 ($P = 0.75$), nor was it between the HAY and CORN group cows on d56 ($P = 0.15$; Figure 2-7A).

GH administration increased liver IGF-I polysomal mRNA abundance in the HAY group cows from d56 to d63 ($P < 0.01$) and also in the CORN group cows from d0 to d7 and from d56 to d63 ($P < 0.01$ and < 0.01, respectively). Liver IGF-I polysomal mRNA on d63 was more abundant in the CORN group cows than the HAY group cows ($P < 0.05$), but its abundance in the CORN group cows was not different between d7 and d63 ($P = 0.99$). The GH-stimulated

increase in liver IGF-I polysomal mRNA from d56 to d63 in the CORN group cows tended to be greater than that in the CORN group cows from d0 to d7 ($P = 0.056$), and was greater than that in the HAY group cows from d56 to d63 ($P < 0.01$; Figure 2-7B). Therefore, increased nutritional intake increased GH-stimulated increase in the liver IGF-I polysomal mRNA abundance, suggesting that increased nutritional intake increased the translational efficiency of IGF-I mRNA in the liver in response to GH administration.

Effects of feeding level and GH administration on serum IGFBP-3 abundance

Serum IGFBP-3 abundance tended to be affected by diet sequences ($P = 0.078$), and there were an effect of day and an interaction of diet sequence with day ($P < 0.01$ and < 0.01, respectively). As shown in Figure 2-8A and 2-8B, serum IGFBP-3 abundance prior to GH administration was not different among d0, d56 and d91 in the HAY group cows ($P = 0.99$, 0.99 and 0.99, respectively), nor was it in the CORN group cows ($P = 0.99$, 0.99 and 0.99, respectively). There was also no difference in serum IGFBP-3 abundance between the two groups of cows on d0, d56 and d91 ($P = 0.86$, 0.86 and 0.98, respectively), or on d14, d49 and d70 ($P = 0.47$, 0.81 and 0.47, respectively). These results indicate that supplementation of concentrates or moderate food restriction did not alter basal serum IGFBP-3 abundance in cows.

GH administration increased serum IGFBP-3 abundance of the HAY group cows from d0 to d7 ($P < 0.05$), but not from d56 to d63 or d91 to d98 ($P = 0.26$ and 0.99, respectively). Growth hormone administration increased serum IGFBP-3 abundance of the CORN group cows from d0 to d7, and from d56 to d63 ($P < 0.05$ and < 0.01, respectively), but not from d91 to d98 ($P = 0.86$). Growth hormone-stimulated serum IGFBP-3 was not different between the HAY and CORN group cows on d1, d3 or d7 ($P = 0.32$, 0.61 and 0.50, respectively), nor was it on d92 and d98 ($P = 0.85$ and 0.10, respectively). However, it was greater in the CORN group cows than in

the HAY group cows on both d59 and d63 ($P < 0.01$ and <0.01, respectively). Growth hormone-stimulated increase in serum IGFBP-3 was not different between the two groups of cows from d0 to d7, or from d91 to d98 ($P = 0.99$ and 0.83, respectively), but it was greater in the CORN group cows than in the HAY group cows from d56 to d63 ($P < 0.01$). Therefore, increased nutritional intake amplified serum IGFBP-3 response to GH administration.

Effects of feeding level and GH administration on liver IGFBP-3 mRNA abundance

Liver IGFBP-3 mRNA abundance was not affected by diet sequences ($P = 0.32$), and there was no interaction of diet sequence with day ($P = 0.27$). There was an effect of day ($P < 0.05$). As shown in Figure 2-9A and 2-9B, basal liver IGFBP-3 mRNA abundance prior to GH administration was not different among d0, d56 and d91 in the HAY group cows ($P = 0.93$, 0.99 and 0.99, respectively) or in the CORN group cows ($P = 0.99$, 0.94 and 0.86, respectively), indicating that supplementation of concentrates or moderate food restriction did not alter basal liver IGFBP-3 mRNA abundance in cows.

Liver IGFBP-3 mRNA abundance was not different between d0 and d7, d56 and d63, or d91 and d98 in the HAY group cows ($P = 0.99$, 0.17 and 0.99, respectively) and in the CORN group cows ($P = 0.99$, 0.77 and 0.97, respectively). Therefore, GH administration did not affect liver IGFBP-3 mRNA abundance.

Effects of feeding level and GH administration on liver IGFBP-3 polysomal mRNA abundance

Liver IGFBP-3 polysomal mRNA abundance was not affected by diet sequences ($P = 0.13$). There was no interaction of diet sequence with day ($P = 0.87$), but there tended to be an effect of day ($P = 0.090$). Basal liver IGFBP-3 polysomal mRNA in the CORN group cows was

not different between d0 on d56 (P = 0.99), nor was it between the HAY and CORN group cows on d56 (P = 0.20; Figure 2-10A).

GH administration did not alter liver IGFBP-3 polysomal mRNA abundance in the HAY group cows from d56 to d63 (P = 0.58), nor did it in the CORN group cows from d0 to d7 or from d56 to d63 (P = 0.99 and 0.43, respectively). The GH-stimulated increase in liver IGFBP-3 polysomal mRNA was not different between that from d56 to d63 and that from d0 to d7 in the CORN group cows (P = 0.61), or between the HAY and CORN group cows from d56 to d63 (P = 0.77; Figure 2-10B). Therefore, both feeding level and GH administration had no effect on liver IGFBP-3 polysomal mRNA abundance.

Effects of feeding level and GH administration on serum ALS abundance

Serum ALS abundance was affected by diet sequences (P < 0.05). There were an effect of day and an interaction between diet sequence and day (P < 0.01 and < 0.05, respectively; Figure 2-11A and 2-11B). Serum ALS abundance in both groups of cows was lower on d56 than d0 (P < 0.01 and < 0.05, respectively), but not different between d56 and d91 (P = 0.65 and 0.76, respectively). Serum ALS abundance was not different between the two groups of cows on d0, d56 or d91 (P = 0.70, 0.31 and 0.38, respectively), nor was it on d49 or d70 (P = 0.55 and 0.21 respectively). These results indicate that supplementation of concentrates or moderate food restriction did not alter basal serum ALS abundance in cows.

GH administration increased serum ALS abundance of the HAY group cows from d56 to d63 (P < 0.01), but not from d0 to d7 or from d91 to d98 (P = 0.32 and 0.22, respectively). Growth hormone also increased serum ALS in the CORN group cows from d56 to d63 and from d91 to d98 (P < 0.01 and < 0.01, respectively), but not from d0 to d7 (P = 0.96). Growth hormone-stimulated serum ALS abundance was not different between the two groups of cows on

d1, d3 or d7 (P = 0.73, 0.87 and 0.58, respectively), but it was more abundant in the CORN group cows than in the HAY group cows on d59, d63 and d98 (P < 0.01, < 0.01 and <0.01 respectively). Growth hormone-stimulated increase in serum ALS abundance was not different between the two groups of cows from d0 to d7 or from d91 to d98 (P = 0.99 and 0.43, respectively), but tended to be greater in the CORN group cows than the HAY group cows from d56 to d63 (P = 0.054). Therefore, increased nutritional intake tended to elevate serum ALS response to GH administration.

Effects of feeding level and GH administration on liver ALS protein abundance

Liver ALS protein abundance was not affected by diet sequences (P = 0.12), and there was no interaction of diet sequence with day (P = 0.18). However, there was an effect of day (P < 0.01). As shown in Figure 2-12A and 2-12B, basal liver ALS protein abundance was not different among d0, d56 and d91 in the HAY group cows (P = 0.99, 0.99 and 0.99, respectively) or in the CORN group cows (P = 0.99, 0.99 and 0.99, respectively). It was also not different between the two groups of cows on d0, d56 or d91 (P = 0.19, 0.43 and 0.84, respectively). These results indicate that supplementation of concentrates or moderate food restriction did not alter basal liver ALS protein abundance in cows.

GH administration increased liver ALS protein abundance in the HAY group cows on d7 (P < 0.01), but not on d63 and d98 (P = 0.22 and 0.81, respectively). Growth hormone administration increased liver ALS protein abundance in the CORN group cows on d7 and d63 (P < 0.01 and < 0.01, respectively), and tended to increase it on d98 (P = 0.072). Liver ALS protein abundance was not different between the two groups of cows on both d7 and d98 (P = 0.24 and 0.60, respectively), but the abundance on d63 was greater in the CORN group cows than in the HAY group cows (P < 0.05). The GH-stimulated increase in liver ALS protein

abundance was not different between the two groups of cows from d0 to d7, from d56 to d63, and from d91 to d98 (P = 0.99, 0.44 and 0.99, respectively). Therefore, increased nutritional intake did not increase liver ALS protein response to GH administration.

Effects of feeding level and GH administration on liver ALS mRNA abundance

Liver ALS mRNA abundance was not affected by diet sequences (P = 0.76), and there was no interaction of diet sequence with day (P = 0.69). However, there was an effect of day (P < 0.01). Prior to GH administration, liver ALS mRNA abundance was not different among d0, d56 and d91 in the HAY group cows (P = 0.99, 0.99 and 0.99, respectively) or in the CORN group cows (P = 0.95, 0.99 and 0.93, respectively). It was also not different between the two groups of cows on d0, d56 and d91 (P = 0.24, 0.28 and 0.77, respectively; Figure 2-13A and 2-13B). These results indicate that supplementation of concentrates or moderate food restriction did not alter basal liver ALS mRNA abundance.

GH administration increased liver ALS mRNA abundance in the HAY group cows on d7 and d56 (P < 0.01 and < 0.05, respectively), but not on d98 (P = 0.48). It also increased ALS mRNA abundance in the CORN group cows on d7 and d63 (P < 0.05 and < 0.01, respectively), but not on d98 (P = 0.27). However, GH-stimulated liver ALS mRNA abundance was not different between the two groups of cows on d7, d63 and d98 (P = 0.81, 0.66 and 0.55, respectively). The GH-stimulated increase in liver ALS mRNA was also not different between the two groups of cows from d0 to d7, from d56 to d63, and from d91 to d98 (P = 0.99, 0.99 and 0.99, respectively). Therefore, GH administration increased liver ALS mRNA abundance, but increased nutritional intake did not affect liver ALS mRNA response to GH administration.

Effects of feeding level and GH administration on liver ALS polysomal mRNA abundance

Liver ALS polysomal mRNA abundance was not affected by diet sequences ($P = 0.57$), and there was no interaction between diet sequence and day ($P = 0.44$). However, there was an effect of day ($P < 0.01$). As shown in Figure 2-14A, prior to GH administration, liver ALS polysomal mRNA in the CORN group cows was not different between d0 and d56 ($P = 0.72$), nor was it between the HAY and CORN group cows on d56 ($P = 0.93$).

GH administration did not alter liver ALS polysomal mRNA abundance in the CORN group cows from d0 to d7 or from d56 to d63 ($P = 0.15$ and 0.15, respectively). Growth hormone administration increased liver ALS polysomal mRNA in the HAY group cows from d56 to d63 ($P < 0.05$). Liver ALS polysomal mRNA abundance in the CORN group cows was not different between d7 and d63 ($P = 0.72$), nor was it between the two groups of cows on d63 ($P = 0.37$). The GH-stimulated increase in liver ALS polysomal mRNA from d56 to d63 in the CORN group cows was not different from that from d0 to d7 ($P = 0.95$), or that from d56 to d63 in the HAY group cows ($P = 0.51$; Figure 2-14B). Therefore, increased nutritional intake did not alter basal or GH-stimulated increase in liver ALS polysomal mRNA abundance.

DISCUSSION

GH-stimulated serum IGF-I concentration in cattle was increased by increased nutritional intake and decreased by food restriction. These results indicate that serum IGF-I response to GH administration is modulated by feeding levels.

One possibility for the greater serum IGF-I under increased nutritional intake is that degradation of IGF-I in serum is reduced when nutritional intake is increased. Our studies indicated that the feeding level-dependent changes in GH-stimulated serum IGF-I are not due to changed degradation of IGF-I in serum.

Another possibility for the greater serum IGF-I response to GH under increased nutritional intake is that GH stimulates greater IGF-I expression in the liver, because approximately 75% of circulating IGF-I is synthesized and secreted from liver, and liver IGF-I mRNA expression is mainly controlled by GH (Donahue and Beamer, 1993; Sjogren et al., 1999). Our results indicated that GH administration increased liver IGF-I mRNA abundance, which is as expected. However, GH-stimulation of liver IGF-I mRNA was not enhanced by increased nutritional intake, suggesting that the greater serum IGF-I response to GH administration under increased nutritional intake is not due to greater liver IGF-I mRNA production in response to GH. However, under increased intake of nutrition, GH caused a greater increase in IGF-I mRNA that was associated with polysomes in the liver, indicating that increased nutritional intake and GH together may increase translation of IGF-I mRNA in the liver. This increase can explain at least in part why under increased nutritional intake GH stimulates a greater increase in serum IGF-I.

We found that GH increased serum IGFBP-3, and that this increase was greater under increased nutritional intake, suggesting that increased nutritional intake and GH together

stimulate greater increase in serum IGFBP-3. Interestingly, both basal and GH-stimulated liver IGFBP-3 mRNA were not affected by feeding level, and feeding level also did not alter the amount of liver IGFBP-3 mRNA associated with polysomes. These results indicated that the greater GH stimulation of serum IGFBP-3 under increased nutritional intake is not due to greater IGFBP-3 mRNA expression or translation in the liver.

Under increased nutritional intake, GH also tended to induce greater increase in serum ALS abundance. However, GH-stimulated increase in liver ALS protein, total mRNA or the amount of ALS mRNA associated with polysomes was not affected by feeding level, indicating that the greater GH-stimulated increase in serum ALS under increased nutritional intake is not due to greater ALS mRNA expression or translation in the liver.

Binary IGF-I / IGFBP-3 and ternary IGF-I / IGFBP-3 / ALS complexes function as a reservoir not only for IGF-I, but also for IGFBP-3 and ALS in the blood (Binoux and Hossenlopp, 1988; Albiston and Herington, 1990; Yakar et al., 2002b; Nindl et al., 2003). ALS-deficient patients or ALS-knockout mice showed marked reductions in serum IGF-1 and IGFBP-3 levels (Boisclair et al., 2000; Haluzik et al., 2003; Hwa et al., 2006; Domene et al., 2007). Overexpression of IGFBP-3 elevated serum IGF-I level (Modric et al., 2001), and overexpression of IGF-I increased serum IGFBP-3 concentration (Camacho-Hubner et al., 1991; Liao et al., 2006). These studies suggest that a change in the concentration of any of IGF-I, IGFBP-3 and ALS in the serum will change the abundance of the binary or ternary complex. In our study, under increased nutritional intake, more liver IGF-I mRNA was associated with polysomes in response to GH administration. This increase in polysomal IGF-I mRNA is expected to contribute to the increased IGF-I production in the liver and hence increased serum IGF-I. Increased serum IGF-I may further bind to unsaturated serum IGFBP-3 and ALS to form

binary and ternary complexes. The increased formation of binary and ternary complexes may in turn increase serum concentrations of IGF-I, IGFBP-3 and ALS, although liver productions of IGFBP-3 and ALS are not changed.

In summary, GH can stimulate growth in cattle and this effect is modulated by feeding level. Increased nutritional intake or food restriction does not change basal serum IGF-I, IGFBP-3 or ALS concentration, or their mRNA expressions in the liver. However, increased nutritional intake increases serum IGF-I concentration response to GH administration. This increase in serum IGF-I response to GH is not due to a reduced degradation of IGF-I in serum, or greater GH-stimulation of liver IGF-I mRNA expression. However, increased nutritional intake and GH together seem to improve translation of IGF-I mRNA in the liver and hence increase serum IGF-I. Under increased nutritional intake, GH stimulates a greater increase in serum IGFBP-3 and also tends to increase serum ALS. These increases in serum IGFBP-3 and ALS are not due to greater production of them in the liver, but may be due to reduced clearance of them from the blood as a result of elevated serum IGF-I and formation of IGF-I / IGFBP-3 and IGF-I / IGFBP-3 / ALS complexes in the blood.

Table 2-1. Primers used in real time-PCR

mRNA	GenBank #	Name	Primer sequence (from 5' to 3')	Product length
IGF-I	BC126802	bIGFIF1	GTTGGTGGATGCTCTCCAGT	148 bp
		bIGFIR1	CTCCAGCCTCCTCAGATCAC	
IGFBP-3	NM_174556	bIGFBP-3-F1	CAGAGCACAGACACCCAGAA	234 bp
		bIGFBP-3-R1	CTGCCCGTACTTATCCACACA	
ALS	DQ444712	bALSqPF1	GCAGGTAACAAGCTGGCCTA	113 bp
		bALSqPR1	ACGAAGACATTGGCCTTGAC	
GAPDH	NM_001034034	bGAPDHF1	GGGTCATCATCTCTGCACCT	176 bp
		bGAPDHR1	GGTCATAAGTCCCTCCACGA	

Figure 2-1. Diagram of experimental design.

Twenty non-lactating and non-pregnant cows were randomly assigned to two pens. One pen of cows, named HAY group cows, had *ad libitum* access to fescue hay from d0 to d98. The other pen of cows, named CORN group cows, had *ad libitum* access to fescue hay from d0 to d7, 9.1 kg of concentrates per cow per day in addition to free access to hay from d8 to d63, and restricted access to hay (90% *ad libitum*) from d64 to d98, respectively. On d0, d56 and d91, each cow was injected with 500 mg of slow-release bovine GH. Blood samples were collected each day during the week of GH administration, and once every week between GH administrations. Liver biopsy was performed on the day before and the 7th day after each GH administration (i.e. on d0, 7, 56, 63, 91 and 98).

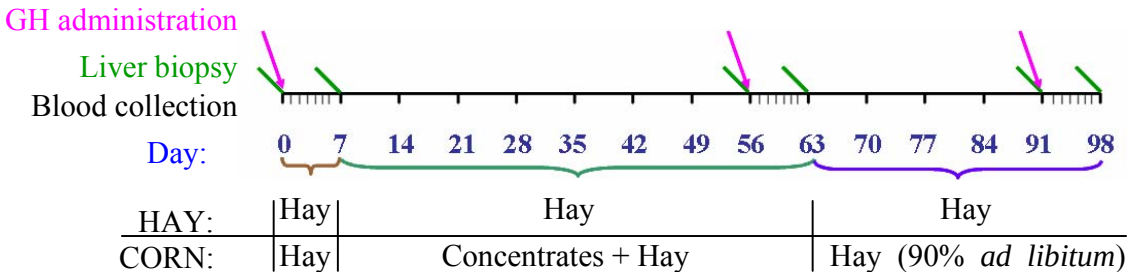


Figure 2-2. Average daily gain of cows.

A: Body weights of the HAY and CORN group cows. The first day of the study was designated day 0 (d0). Data = LS mean ± SE (n = 10). **B:** Average daily gain (ADG) during different time periods, that is, from d0 to d7, from d7 to d56, from d56 to d63, from d63 to d91 and from d91 to d98. Each bar is LS mean ± SE (n = 10). ** $P < 0.01$: CORN vs. HAY during the same time period. There were effects of diet sequence and time period, and an interaction of diet sequence with time period ($P < 0.01$, < 0.01 and < 0.01, respectively). The ADG of the CORN group cows from d56 to d63 was greater than that from d0 to d7, from d7 to d56, from d63 to d91 or from d91 to d98 ($P < 0.01$, < 0.01, < 0.01 and < 0.01, respectively). The ADG of the CORN group cows from d91 to d98 was greater than that from d63 to d91 ($P < 0.01$). The ADG of the CORN group cows from d7 to d56 was greater than that from d63 to d91 ($P < 0.01$).

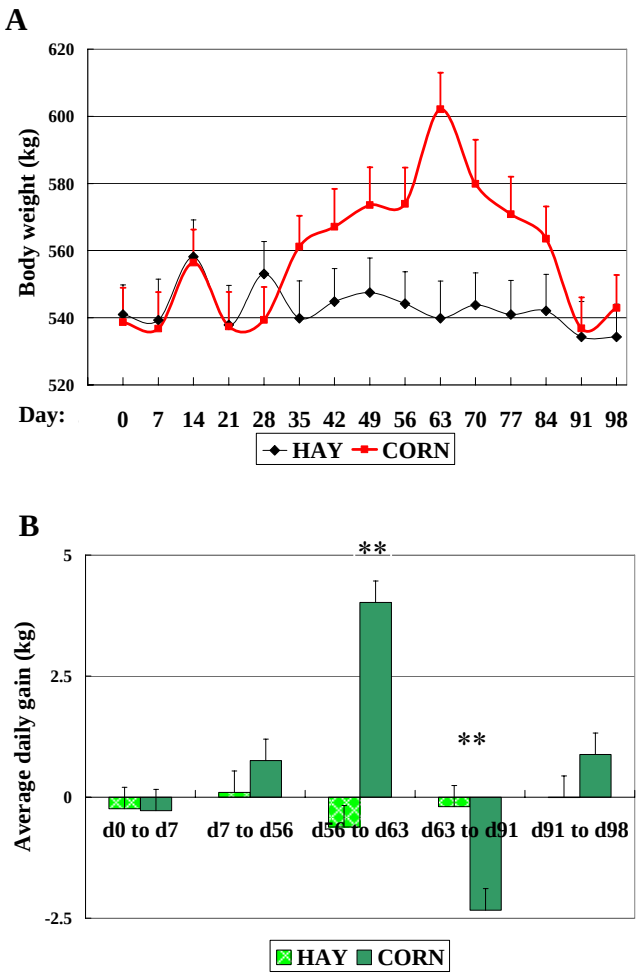


Figure 2-3. Serum IGF-I concentrations in cows.

Serum IGF-I concentrations (ng/mL) of five cows from each group were determined by radioimmunoassay. Data = LS mean ± SE (n = 5). There were effects of diet sequence, day and an interaction between diet sequence and day ($P < 0.01$, < 0.01 and < 0.01, respectively). Serum IGF-I concentrations were increased after each GH administration ($P < 0.01$). ** $P < 0.01$, CORN versus HAY on the same individual day.

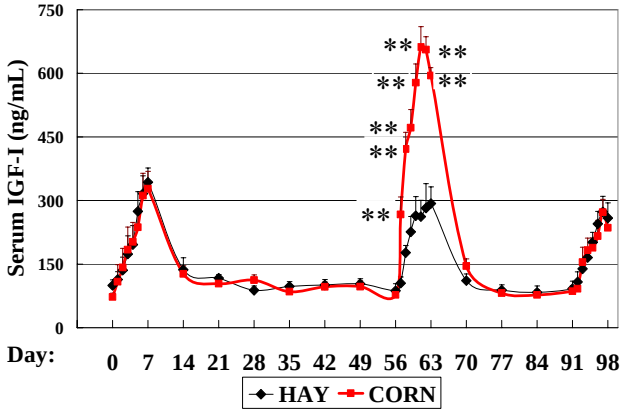


Figure 2-4. Serum growth hormone concentrations in cows.

Serum GH concentrations (ng/mL) of five cows from each group were determined by radioimmunoassay. Data = LS mean ± SE (n = 5). There were no effects of diet sequence and an interaction between diet sequence and day (P = 0.48 and 0.93, respectively). There was an effect of day (P < 0.01).

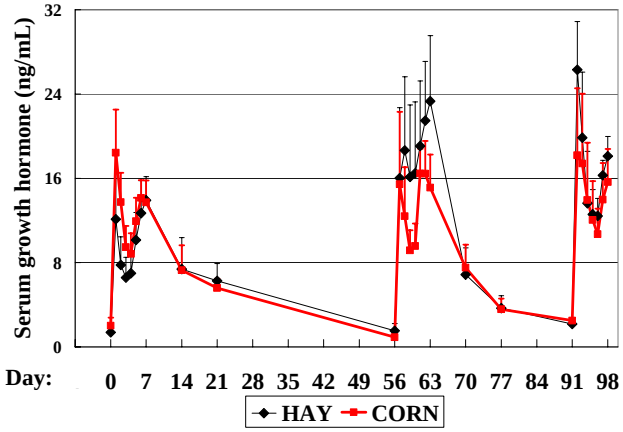


Figure 2-5. ^{125}I-IGF-I stability in cow serum.

^{125}I-IGF-I was incubated at 37 °C in the serum of five cows from each group or H_2O (negative control) for 12 h, and then resolved by SDS-PAGE. **A:** Images of undegraded ^{125}I-IGF-I. **B:** Densitometric quantification of undegraded ^{125}I-IGF-I in panel A. Data = LS mean ± SE (n = 5). There were no effects of diet sequence and an interaction between diet sequence and day (P = 0.99 and 0.92, respectively). There was an effect of day (P < 0.01).

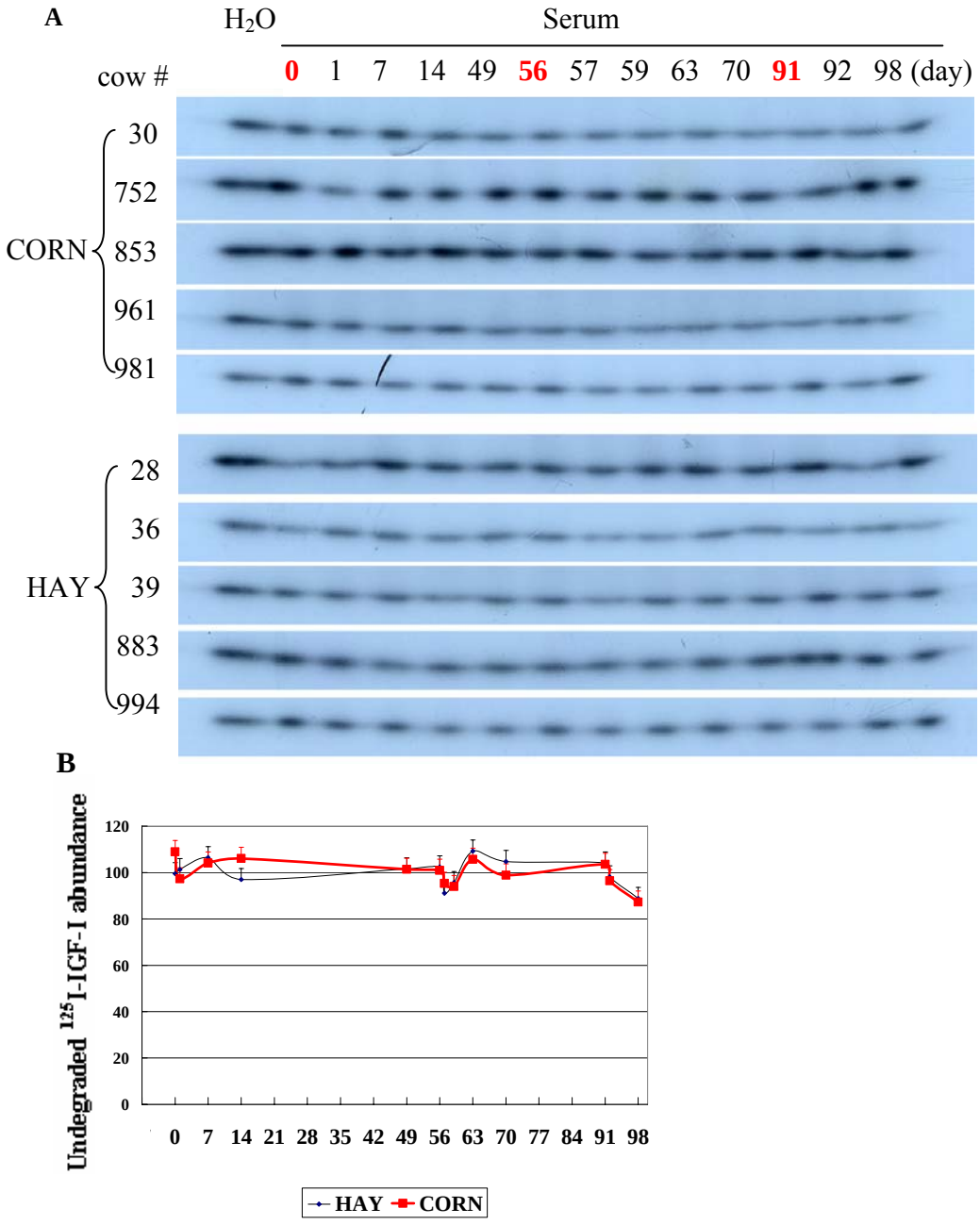
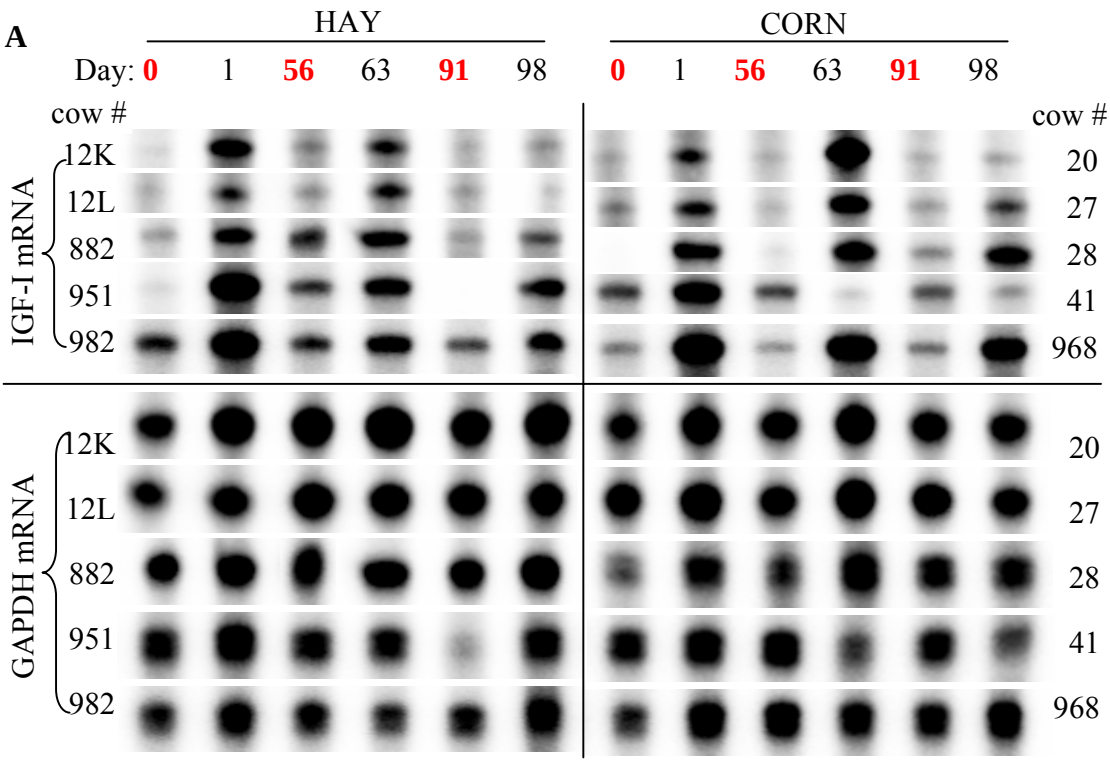
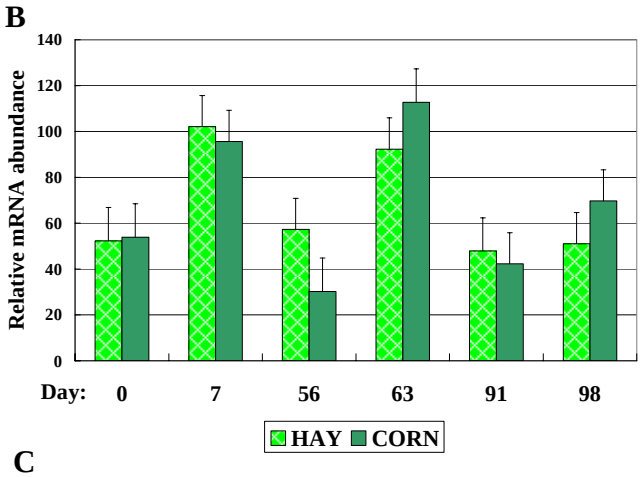


Figure 2-6. Liver IGF-I mRNA abundance in cows.

Liver total RNA of five cows from each group was hybridized with ^{32}P-labeled probes for bovine IGF-I and GAPDH mRNAs, digested by RNase A/T1, and then resolved by gel electrophoresis. **A:** RPA images of liver total IGF-I and loading control GAPDH mRNAs. The ribonuclease-protected RNA fragments were indicated. **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of IGF-I band was normalized to that of GAPDH band in the same sample. There was no difference in the abundance of GAPDH mRNA between the HAY and CORN group cows ($P = 0.99$). Each bar represents LS mean ± SE ($n = 5$). There were no effects of diet sequence and an interaction between diet sequence and day ($P = 0.98$ and 0.11, respectively). There was an effect of day ($P < 0.01$). Liver IGF-I mRNA in the HAY group cows on d7 was more abundant than on d0 ($P < 0.01$). Liver IGF-I mRNA in the CORN group cows on d7 and d63 was more abundant than on d0 and d56, respectively ($P < 0.05$ and < 0.01, respectively). **C:** GH-stimulated increase in liver IGF-I mRNA from d0 to d7, from d56 to d63 and from d91 to d98 (i.e., d7-d0, d63-d56 and d98-d91).


A
HAY
CORN
Day: 0 1 56 63 91 98 0 1 56 63 91 98
cow #
cow #
IGF-I mRNA
GAPDH mRNA
12K
12L
882
951
982
20
27
28
41
968


B
Relative mRNA abundance
140
120
100
80
60
40
20
0
Day: 0 7 56 63 91 98
HAY CORN

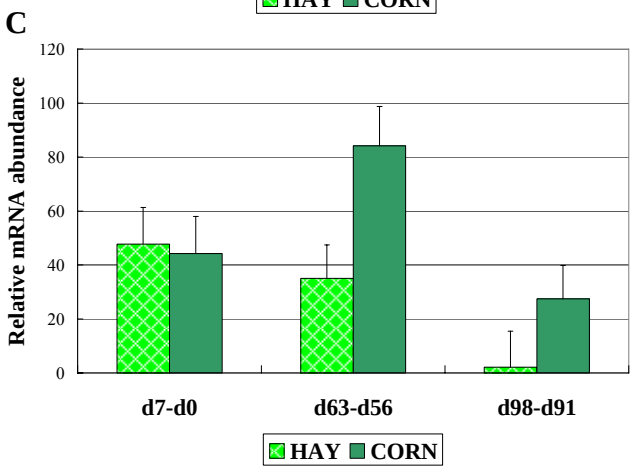

C
Relative mRNA abundance
120
100
80
60
40
20
0
d7-d0
d63-d56
d98-d91
HAY CORN

Figure 2-7. Liver IGF-I polysomal mRNA abundance in cows.

Liver IGF-I polysomal mRNA of five cows from each group was quantified by real time-PCR. **A:** Relative abundance of liver IGF-I polysomal mRNA. Each bar represents LS mean ± SE (n = 5). There was no effect of diet sequence (P = 0.47). There were effects of day and an interaction between diet sequence and day (P < 0.01 and <0.05, respectively). After 7 days of GH administration, liver IGF-I polysomal mRNA abundance was increased in all diet sequences (P < 0.01). * P < 0.05, CORN versus HAY on the same individual day. **B:** GH-stimulated increase in liver polysomal mRNA in cows. On the x-axis, HAY d63-d56, CORN d7-d0 and CORN d63-d56 mean the difference in IGF-I polysomal mRNA between d63 and d56 in the HAY group cows, d7 and d0 in the CORN group cows, and d63 and d56 in the CORN group cows, respectively. Each bar represents LS mean ± SE (n = 5). Means with different letters differ (P < 0.01).

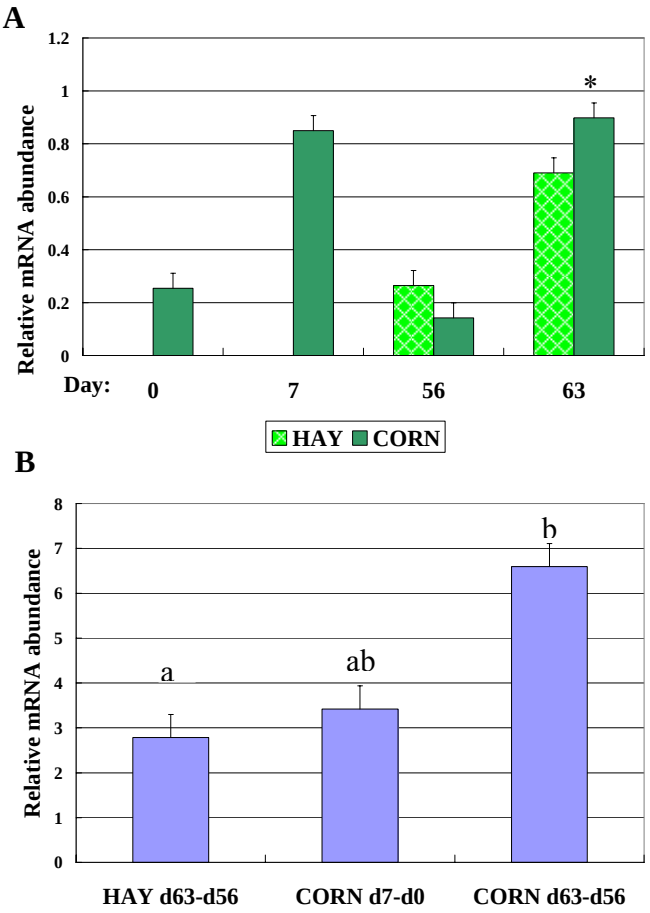
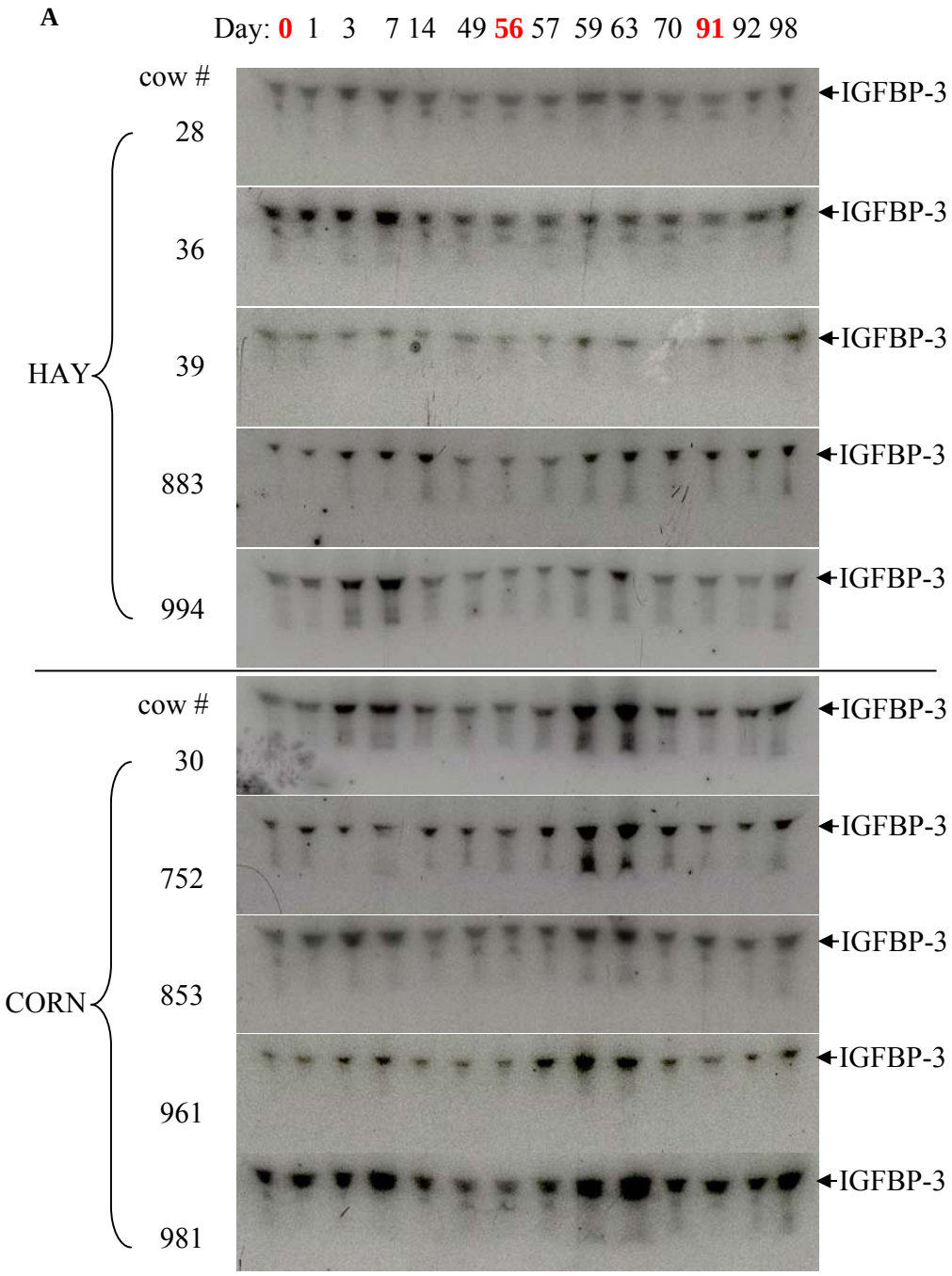


Figure 2-8. Serum IGFBP-3 protein abundance in cows.

Sera of 5 five cows from each group were resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane, and then blotted with ^{125}I-labeled IGF-I. **A:** Images of Western ligand blotting analysis of serum IGFBP-3. **B:** Densitometric quantification of IGFBP-3 in panel A. Data = LS mean ± SE (n = 5). There tended to be an effect of diet sequence ($P = 0.078$). There were effects of day and an interaction between diet sequence and day ($P < 0.01$). Growth hormone administration increased serum IGFBP-3 abundance in the HAY group cows from d0 to d7 ($P < 0.05$), and in the CORN group cows from both d0 to d7 and d56 to d63 ($P < 0.05$ and < 0.01, respectively). ** $P < 0.01$, CORN versus HAY on the same individual day.


A
Day: 0 1 3 7 14 49 56 57 59 63 70 91 92 98
cow #
28
36
39
883
994
HAY
IGFBP-3
IGFBP-3
IGFBP-3
IGFBP-3
IGFBP-3
cow #
30
752
853
961
981
CORN
IGFBP-3
IGFBP-3
IGFBP-3
IGFBP-3
IGFBP-3

B

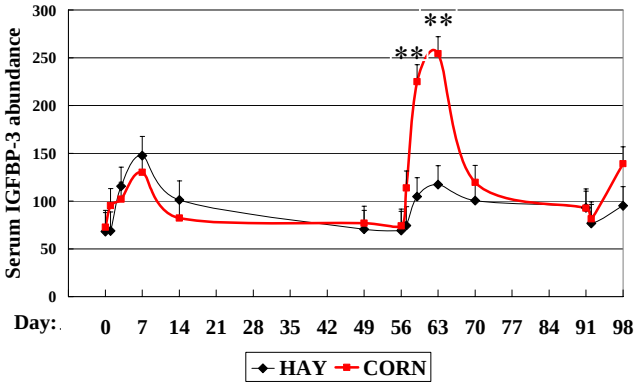
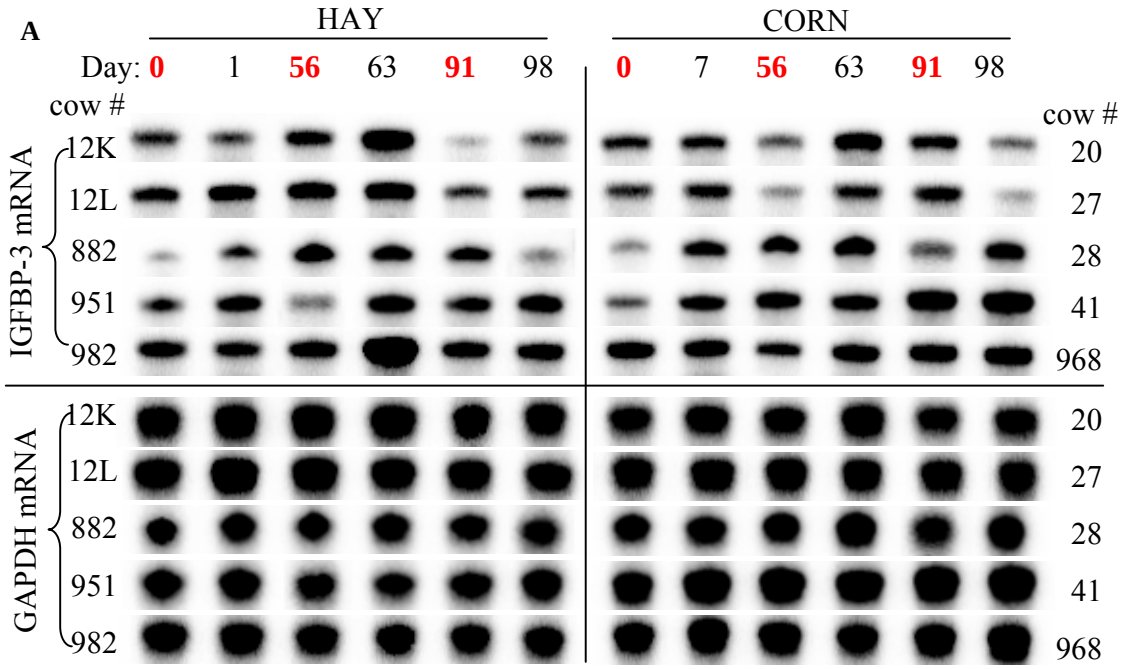

Serum IGFBP-3 abundance
300
250
200
150
100
50
0
**
**
Day:
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98
HAY
CORN

Figure 2-9. Liver IGFBP-3 mRNA abundance in cows.

Liver total RNA of five cows from each group was hybridized with α-^{32}P-labeled probes for bovine IGFBP-3 and GAPDH mRNAs, digested by RNase A/T1, and then resolved by gel electrophoresis. **A:** RPA images of liver IGFBP-3 and loading control GAPDH mRNAs. **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of IGFBP-3 band was normalized to that of GAPDH band in the same sample. There was no difference in the abundance of GAPDH mRNA between the HAY and CORN group cows (P = 0.36). Each bar represents LS mean ± SE (n = 5). There were no effects of diet sequence and the interaction between diet sequence and day (P = 0.32 and 0.27, respectively). There was an effect of day (P < 0.05).



B

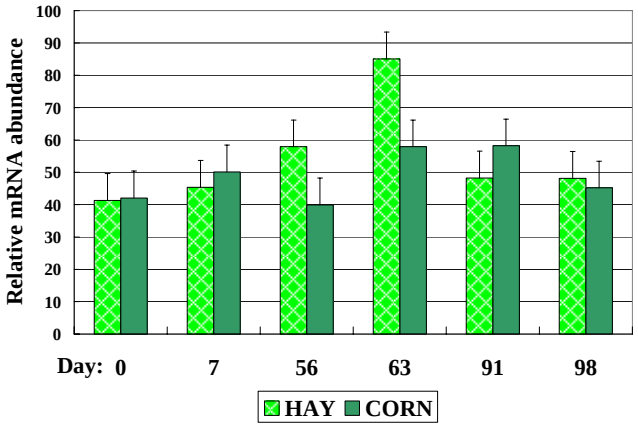
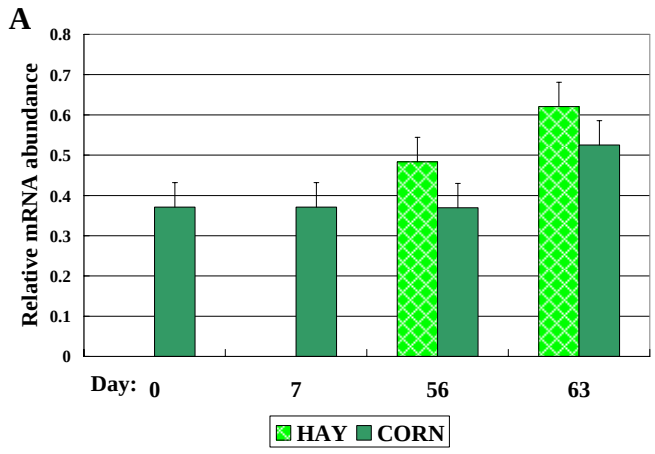

Relative mRNA abundance
100
90
80
70
60
50
40
30
20
10
0
Day: 0
7
56
63
91
98
HAY
CORN

Figure 2-10. Liver IGFBP-3 polysomal mRNA abundance in cows.

Liver IGFBP-3 polysomal mRNA of five cows from each group was quantified by real time-PCR. **A:** Relative abundance of liver IGFBP-3 polysomal mRNA. Each bar represents LS mean ± SE (n = 5). There were no effects of diet sequence and an interaction between diet sequence and day (P = 0.13 and 0.87, respectively). There tended to be an effect of day (P = 0.090). **B:** GH-stimulated increase in liver polysomal mRNA in cows. On the x-axis, HAY d63-d56, CORN d7-d0 and CORN d63-d56 mean the difference in IGFBP-3 polysomal mRNA between d63 and d56 in the HAY group cows, d7 and d0 in the CORN group cows, and d63 and d56 in the CORN group cows, respectively. Each bar represents LS mean ± SE (n = 5).



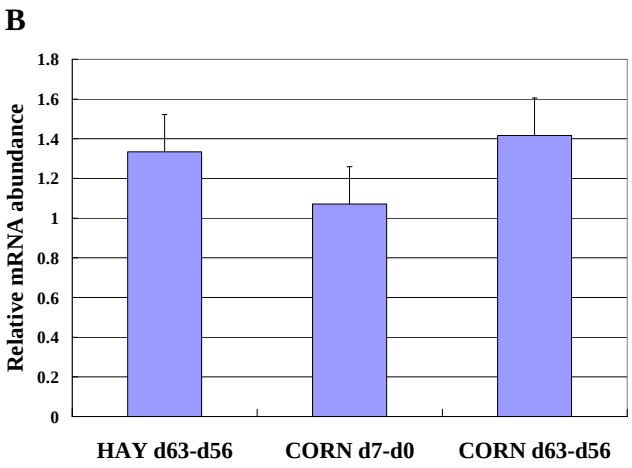
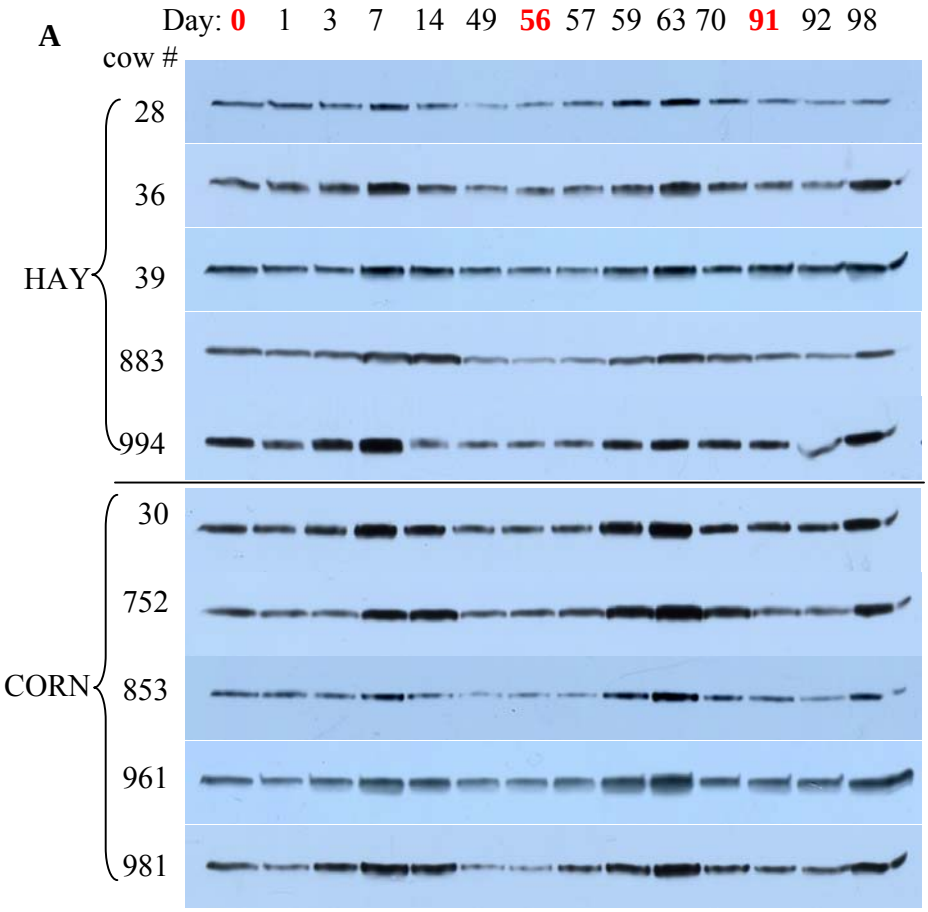
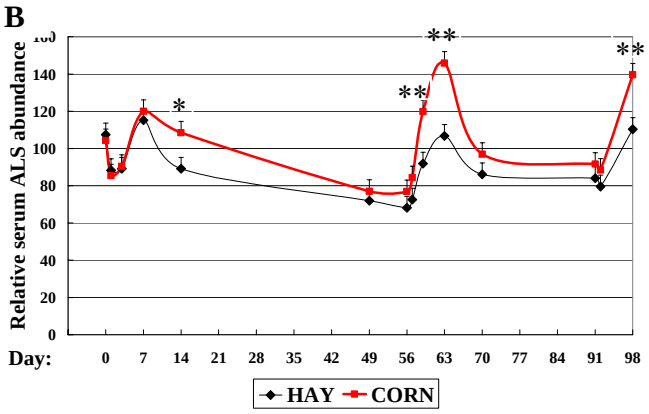


Figure 2-11. Serum ALS protein abundance in cows.

Serum sample of five cows from each group was resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane, and then blotted with anti-ALS antibody. **A:** Images of Western blot analysis of serum ALS protein. **B:** Densitometric quantification of bands in panel A. Each bar is LS mean ± SE (n = 5). There were effects of diet sequence, day and an interaction between diet sequence and day ($P < 0.05$, < 0.01 and < 0.05, respectively). Serum ALS protein abundance was less on d56 than d0 in both the HAY and CORN group cows ($P < 0.01$ and < 0.05, respectively). Growth hormone administration increased serum ALS protein abundance in the HAY group cows from d56 to d63 ($P < 0.01$), and in the CORN group cows from d56 to d63 and from d91 to d98 ($P < 0.01$ and < 0.01, respectively). * $P < 0.05$ and ** $P < 0.01$, CORN versus HAY on the same individual day. **C:** Images of polyacrylamide gels that were stained with GelCode Blue Stain Reagent (PIERCE, Rockford, IL) after electro-transference to show equal protein loading and transfer. M: protein molecular marker.


A
Day: 0 1 3 7 14 49 56 57 59 63 70 91 92 98
cow #
HAY
28
36
39
883
994
CORN
30
752
853
961
981


B
Relative serum ALS abundance
Day:
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98
HAY
CORN

C

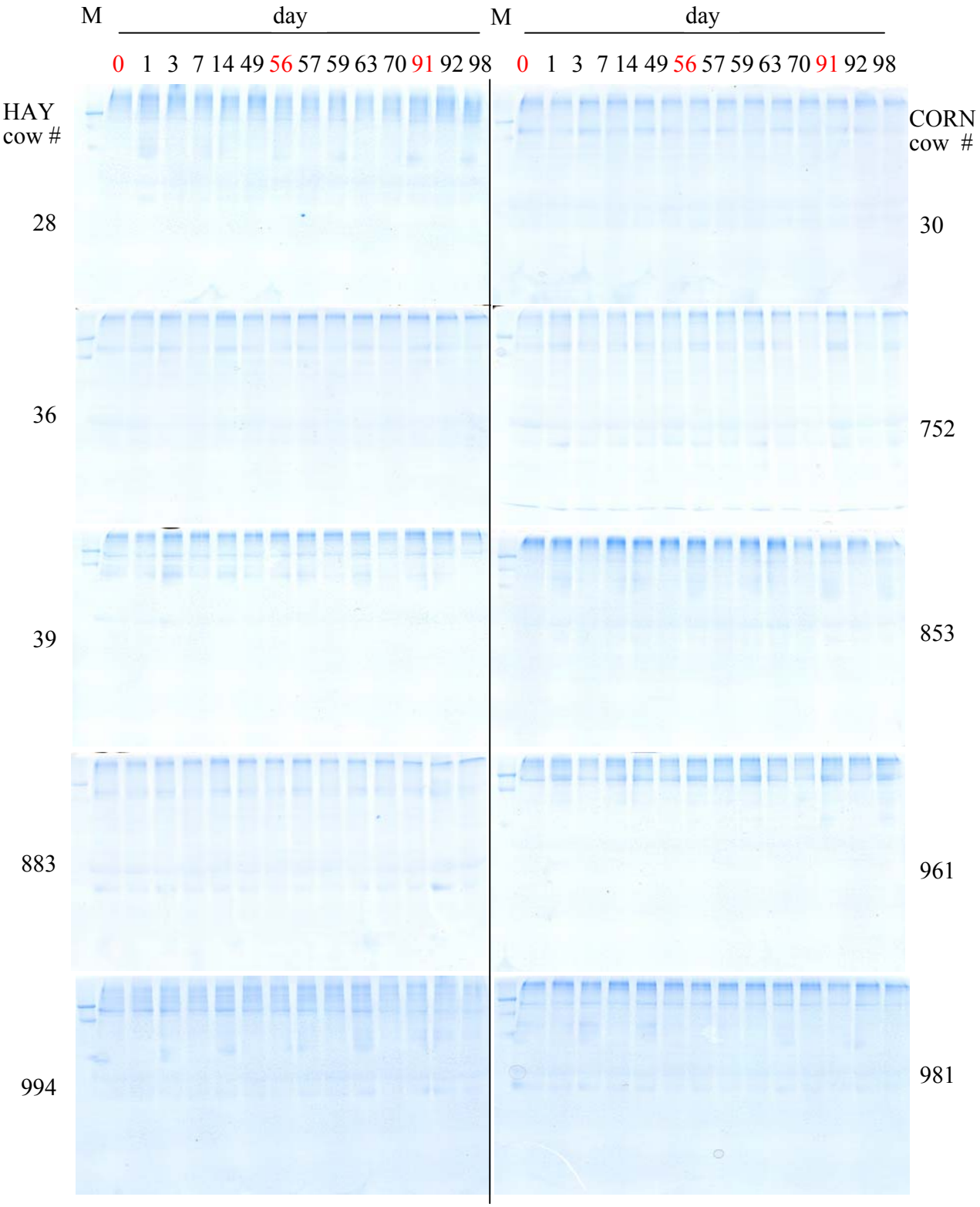
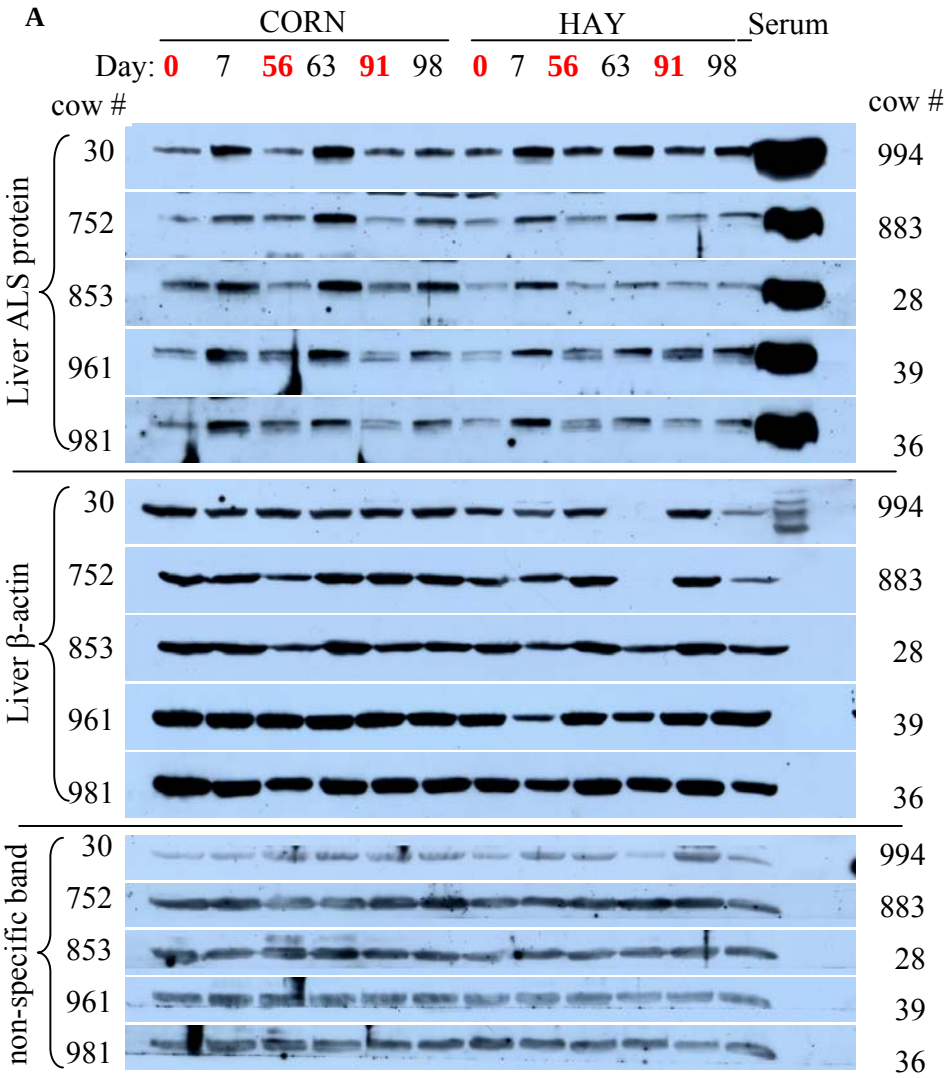
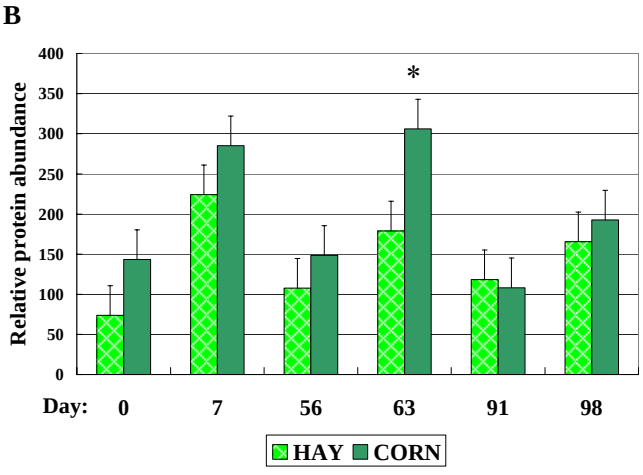

M
day
0 1 3 7 14 49 56 57 59 63 70 91 92 98
M
day
0 1 3 7 14 49 56 57 59 63 70 91 92 98
HAY
cow #
28
36
39
883
994
CORN
cow #
30
752
853
961
981

Figure 2-12. Liver ALS protein abundance in cows.

Liver whole cell lysates of five cows from each group were resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane, and then blotted with anti-ALS and anti-β-actin antibodies. **A:** Images of Western blot analyses. ALS, β-actin and non-specific bands from the stained polyacrylamide gel were indicated. **B:** Densitometric quantification of bands in panel A. Each bar is LS mean ± SE (n = 5). There were no effects of diet sequence or an interaction between diet sequence and day (P = 0.12 and 0.18 respectively). There was an effect of day (P < 0.01). Seven days of GH administration increased liver ALS protein abundance in the HAY group cows on d7 (P < 0.01), and in the CORN group cows on both d7 and d63 (P < 0.01 and < 0.01, respectively). * P < 0.05, CORN versus HAY on the same individual day. **C:** The polyacrylamide gels that were stained with GelCode Blue Stain Reagent (PIERCE, Rockford, IL) after electro-transference were used to show equal protein loading and transfer. M: protein molecular marker.


A
CORN
HAY
Serum
Day: 0 7 56 63 91 98 0 7 56 63 91 98
cow #
cow #
Liver ALS protein
30
752
853
961
981
994
883
28
39
36
Liver β-actin
30
752
853
961
981
994
883
28
39
36
non-specific band
30
752
853
961
981
994
883
28
39
36


B
Relative protein abundance
400
350
300
250
200
150
100
50
0
*
Day: 0 7 56 63 91 98
HAY CORN

C

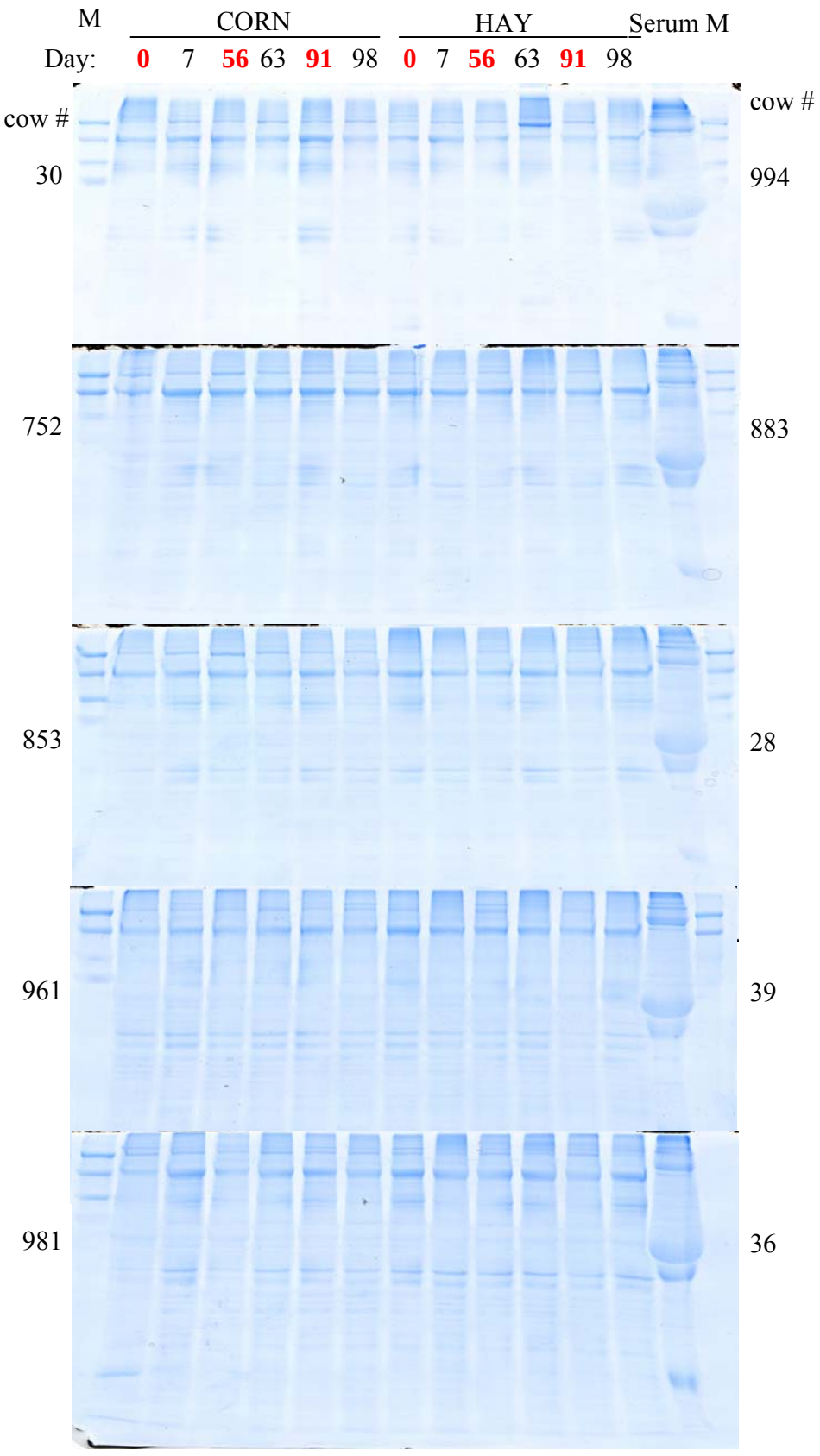
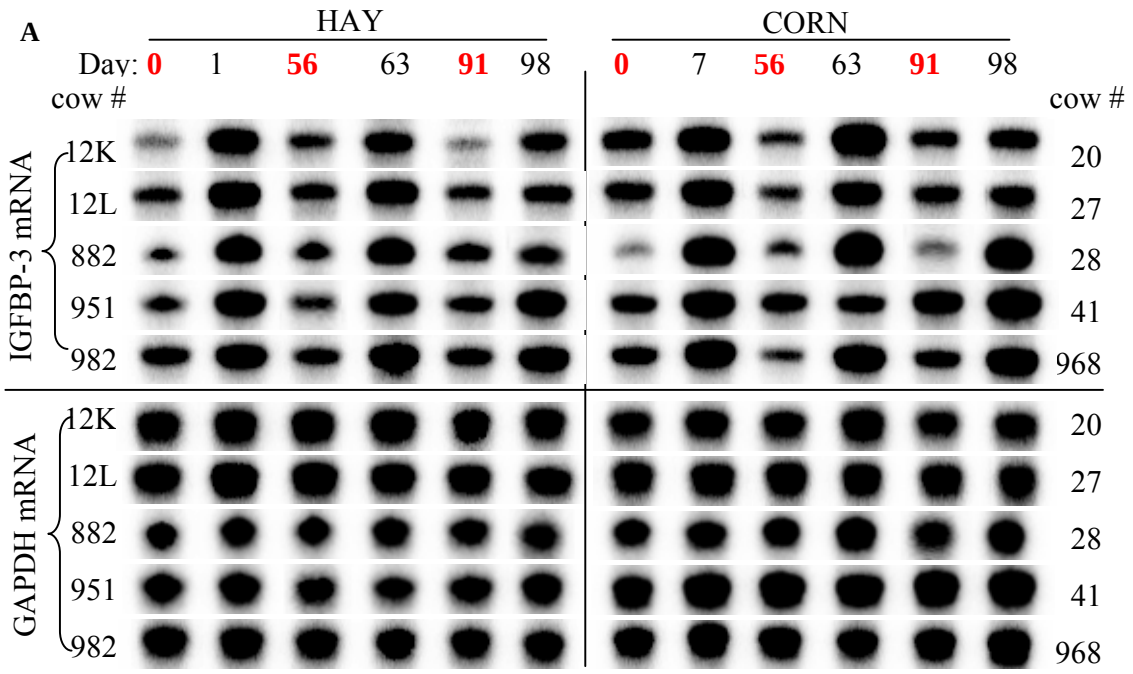

M
CORN
HAY
Serum M
Day: 0 7 56 63 91 98 0 7 56 63 91 98
cow #
30
752
853
961
981
cow #
994
883
28
39
36

Figure 2-13. Liver ALS mRNA abundance in cows.

Liver total RNA of five cows from each group was hybridized with ^{32}P-labeled probes for bovine ALS and GAPDH mRNAs, digested by RNase A/T1, and then resolved by gel electrophoresis. **A:** RPA images of liver ALS and loading control GAPDH mRNAs. The ribonuclease-protected RNA fragments were indicated. **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of ALS band was normalized to that of GAPDH band in the same sample. There was no difference in the abundance of GAPDH between the HAY and CORN group cows (P = 0.36). Each bar represents LS mean ± SE (n = 5). There were no effects of diet sequence and an interaction between diet sequence and day (P = 0.76 and 0.69, respectively). There was an effect of day (P < 0.01). Seven days of GH administration increased liver ALS protein abundance in the HAY group cows on both d7 and d63 (P < 0.01 and < 0.05, respectively), and in the CORN group cows on both d7 and d63 (P < 0.05 and < 0.01, respectively).



B

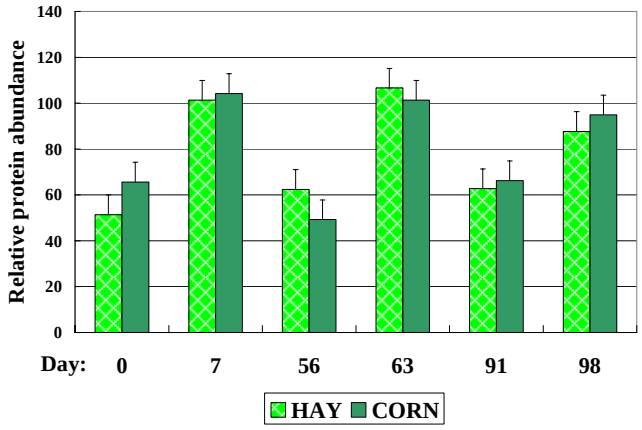
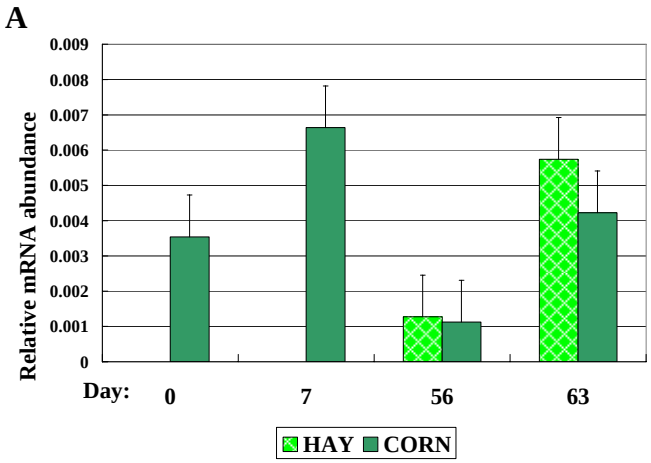
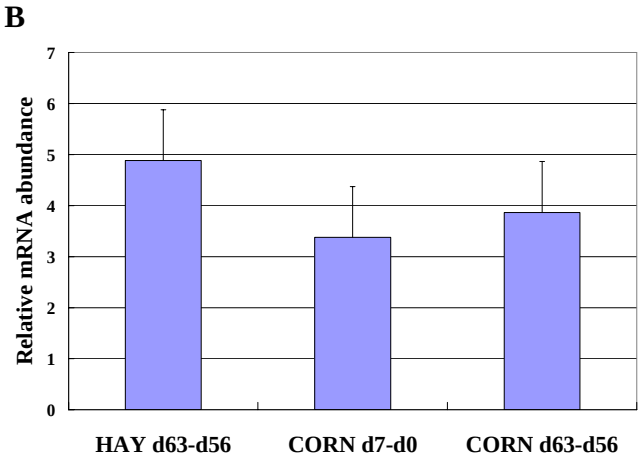

Relative protein abundance
140
120
100
80
60
40
20
0
Day:
0
7
56
63
91
98
HAY
CORN

Figure 2-14. Liver ALS polysomal mRNA abundance in cows.

Liver ALS polysomal mRNA of five cows from each group was quantified by real time-PCR. **A:** Relative abundance of liver ALS polysomal mRNA. Each bar represents LS mean ± SE (n = 5). There were no effects of diet sequence and an interaction between diet sequence and day (P = 0.57 and 0.44, respectively). There was an effect of day (P < 0.01). Seven days of GH administration increased liver ALS protein abundance in the HAY group cows on both d63 (P < 0.05). **B:** GH-stimulated increase in liver ALS polysomal mRNA in cows. On the x-axis, HAY d63-d56, CORN d7-d0 and CORN d63-d56 mean the difference in ALS polysomal mRNA between d63 and d56 in the HAY group cows, d7 and d0 in the CORN group cows, and d63 and d56 in the CORN group cows, respectively. Each bar represents LS mean ± SE (n = 5).





Chapter III

Effects of food deprivation on serum IGF-I concentration in steers and the underlying mechanisms

ABSTRACT

Insulin-like growth factor-I (IGF-I) is important for animal growth and cellular metabolism. This study was conducted to determine the effect of food deprivation on serum IGF-I concentration in cattle and the underlying mechanisms. Five yearling steers were deprived of food for 60 h, while the other five were allowed *ad libitum* access to pasture. A blood sample and subsequently liver, subcutaneous adipose and skeletal muscle biopsy samples were taken from each steer at the end of the 60-h period. Serum IGF-I concentration was decreased by 46% in food-deprived steers ($P < 0.01$). The decrease was unlikely due to increased IGF-I degradation because ^{125}I-labeled IGF-I was equally stable in fed and food-deprived steer sera ($P = 0.98$). Food deprivation decreased liver IGF-I mRNA level by 82% ($P < 0.01$), and this decrease was associated with decreases of total GH receptor (GHR) mRNA ($P < 0.01$), GHR mRNA variant 1A ($P < 0.01$), total GHR protein ($P < 0.05$) and microsomal membrane mature GHR ($P < 0.01$) in the liver. There were no differences in levels of signal transducer and activator of transcription-5 (STAT5), hosphor-STAT5, Janus kinase-2 (JAK2) or hosphor-JAK2 in the liver between fed and food-deprived steers. The mRNA of two negative regulatory factors of the GHR signaling pathway, suppressor of cytokine signaling-2 (SOCS2) and cytokine-inducible SH2 protein (CIS), was increased in the liver of food-deprived steers ($P < 0.05$). Serum IGF binding protein-3 (IGFBP-3) level was decreased by 38% in food-deprived steers ($P < 0.05$), and this decrease was associated with a 59% decrease in liver IGFBP-3 mRNA ($P < 0.05$). Acid labile

subunit (ALS) mRNA was lowered 63% in the liver from food-deprived steers ($P < 0.001$), whereas both liver and serum ALS protein levels were not different between fed and food-deprived steers. These results indicate that multiple mechanisms mediate the decrease in circulating IGF-I concentration during food deprivation, including decreased IGF-I gene expression, and increased clearance of IGF-I from blood.

Keywords: Food deprivation, Insulin-like growth factor-I, IGF binding protein-3, Acid labile subunit, Growth hormone receptor, Suppressor of cytokine signaling-2, Cytokine-inducible SH2 protein, Cattle

INTRODUCTION

Insulin-like growth factor-I (IGF-I), a polypeptide growth factor, is important for somatic growth and metabolism. The IGF-I functions as both an endocrine and paracrine factor (D'Ercole et al., 1984). Liver-specific IGF-I gene inactivation in mice indicated that 75% of circulating IGF-I comes from liver (Sjogren et al., 1999) and liver-derived IGF-I is not essential for postnatal growth, as used to be believed (Le Roith et al., 2001), but is important for normal carbohydrate and lipid metabolism (Sjogren et al., 2001). Several signaling pathways are responsible for IGF-I expression and secretion, including the JAK-STAT pathway, the phosphoinositide-3 kinase(PI3K)-dependent pathway and the mitogen-activated protein kinase (MAPK)-dependent pathway (Davey et al., 2001; Shoba et al., 2001; Lee et al., 2007). The JAK2-STAT5 pathway seems to be most responsible for GH-stimulated IGF-I gene transcription (Davey et al., 2001; Woelfle and Rotwein, 2004; Hwa et al., 2005). Several negative regulatory proteins, including SOCS2 and CIS, down-regulate GHR signaling pathway and IGF-I expression (Matsumoto et al., 1997; Metcalf et al., 2000).

Serum IGF-I concentration is also regulated by IGF-I related peptides, including IGF binding proteins (IGFBP-1 through IGFBP-6) (Albiston and Herington, 1990; Yakar et al., 2002b) and acid labile subunit (ALS). Under normal physiological conditions, less than 5% of IGF-I is circulating as free IGF-I in the bloodstream. Most of serum IGF-I is bound to IGFBP-3 to form a binary complex of IGF-I/IGFBP-3 that functions as vehicle for IGF-I to be delivered to the IGF-I-target tissues, or a ternary complex of IGF-I/IGFBP-3/ALS that functions as a reservoir for circulating IGF-I, decreases the clearance of IGF-I from blood and hence prolongs the half-life of IGF-I in the circulation (Binoux and Hossenlopp, 1988; Albiston and Herington, 1990; Yakar et al., 2002b).

Serum IGF-I is decreased and GH is increased under food deprivation (Zhang et al., 1998; Lee et al., 2002; Wang et al., 2003). The changes in IGF-I and GH concentration under food deprivation seem to reduce anabolic activity and increase catabolism, saving energy for life maintenance (Zhang et al., 2001a; Bauer et al., 2004; Wang et al., 2006). The decrease in serum IGF-I was found to be associated with decreased expression of liver IGF-I and GHR mRNAs (Wang et al., 2003). The exact mechanism by which liver IGF-I gene expression is decreased under food deprivation is not understood. Based on the liver-specific IGF-I gene inactivation studies (Sjogren et al., 1999), 25% of the circulating IGF-I comes from tissues other than the liver, so it is not clear whether decreased productions of IGF-I in non-liver tissues also contribute to the decrease in serum IGF-I under food deprivation. It is also not clear whether serum IGF-I degradation and IGF-I clearance are increased during food deprivation. This study was conducted to address these questions in cattle.

MATERIALS AND METHODS

Experimental design and collection of samples

Ten yearling Holstein steers were randomly assigned to two groups, five steers per group. One group of steers was deprived of food for 60 h (Food-deprived group), while the other had free access to pasture (Fed group). At the end of the 60-h period, a blood sample from each steer was collected from the jugular vein using a 9-mL vacuum tube containing serum clot activator (Greiner bio-one VACUETTE America, Monroe, North Carolina). A few minutes after the blood sampling, biopsies of liver, skeletal muscle and subcutaneous adipose tissues were taken from each animal as described previously (Oxender et al., 1971). Tissue samples were immediately frozen in liquid nitrogen and stored at –80 °C. Blood samples were allowed to clot at room temperature for 1 h, and then centrifuged at 1,800 × g at 4 °C for 25 min to separate serum, which was stored at –20 °C for future assays. The animal-related protocol was approved by the Virginia Tech Animal Care Committee.

Radioimmunoassay (RIA)

Concentrations of serum total IGF-I were determined by radioimmunoassay following an acid-ethanol cryoprecipitation extraction to remove IGF binding proteins (Purup et al., 1993). One hundred microliters of serum sample were mixed with 900 μL extraction solution (87.5% ethanol and 12.5% of 2 N HCl). After 1 h of incubation at room temperature, the mixed solution was centrifuged at 13,000 × g for 10 min at 4 °C, and 500 μL of the supernatant were neutralized by 200 μL of 0.855 *M* Tris base. After 1 h of incubation at – 20 °C, the neutralized solution was centrifuged at 3,000 × g for 30 min, and 30 μL of the supernatant were used for each RIA. One hundred microliters of ^{125}I-labeled IGF-I (2.77×10^4 dpm) and 100 μL of the mouse antiserum raised against bovine IGF-I (a gift of Dr. Bernard Laarveld, University of Saskatchewan) were

added to the 30 μL extracted solution. After incubation for 24 h, 100 μL of the goat-anti-mouse second antiserum (Sigma Chemical Company, St. Louis, MO, USA) were added to each tube. After incubation at 4 °C for 72 h, 1 mL of phosphate-buffered saline (PBS) was added to each tube, and the tube was then centrifuged at 1,560 × g for 30 min at 4 °C. The supernatant were decanted and the radioactivity of the pellet that remained in the tube was measured by gamma counting. The detection range of the RIA was 23.3 to 2,986.7 ng/mL, and the intra-assay coefficient of variation was 9.1%.

Stability assay of ^{125}I-labeled IGF-I in serum

An *in vitro* method was developed to test the stability of ^{125}I-labeled IGF-I in serum. Five microliters of ^{125}I-labeled IGF-I (1.52×10^5 dpm) were incubated at 37 °C for 6 h or 24 h with 50 μL of different proteases: 10 μg/μL of proteinase K (Fisher Scientific, Fair Lawn, NJ), 20 U/μL of collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) and 0.25% Trypsin/2.21 m*M* EDTA in Hank's Balanced Salt Solution (HBSS; Mediatech, Herndon, VA). Five microliters of ^{125}I-labeled IGF-I were incubated at 37 °C in 50 μL of H_2O for 46 h as a negative control. Five microliters of ^{125}I-labeled IGF-I were incubated with 50 μL of serum at 37 °C for 0 h, 28 h, 31 h or 46 h to determine the optimal incubation time for serum. Fifty microliters of serum from each steer were incubated with 5 μL of ^{125}I- IGF-I at 37 °C for 45 h. Following the incubation, 5.5 μL of the solution were resolved by electrophoresis on 8% SDS-polyacrylamide gels at 150 volt for 1.5 h. After electrophoresis, the gels were dried for 1 h on a Model 583 gel dryer (Bio-Rad Laboratories, Hercules, CA) and exposed to phosphor screens. The exposed phosphor screens were scanned on a Molecular Imager FX System (Bio-Rad Laboratories, Hercules, CA). The densities of remained ^{125}I-IGF-I bands were quantified using the ImageJ program (http://rsb.info.nih.gov/ij/index.html).

RNA extraction and quantification

Total RNA from bovine tissues was isolated using TRI Reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. Briefly, frozen tissue (about 0.5 g) was homogenized in 5 mL of TRI Reagent at 20,000 rpm for 45 sec using a Virtis homogenizer model 225318 (Virtis, Gardiner, NY). One milliliter of chloroform was added to the homogenate, and then 2.5 mL of isopropanol were added to the collected aqueous supernatant. Total RNA was collected by centrifugation at 12,000 × g for 10 min at 4 °C, and redissolved in diethypyrocarbonate (DEPC)-treated water. RNA concentrations were measured based on the absorbance at 260 nm in a Spectrophotometer U-2810 (Digilab Hitachi, San Jose, CA), and RNA intactness was verified by electrophoresis on 1% agarose gels containing 20% of formaldehyde (v/v).

Ribonuclease protection assay (RPA)

The plasmid for synthesis of total IGF-I mRNA detection probe was a pGEM-T Easy plasmid containing a 200-bp cDNA insert that corresponded to 137 bp of exon 3 and 63 bp of exon 4 of the bovine IGF-I gene (Kobayashi et al., 1999). The plasmid for bovine total GHR (totGHR) mRNA probe synthesis was a pGEM-T Easy plasmid containing a 317-bp cDNA insert that was composed of 81 bp of exon 2, 66 bp of exon 3, 131 bp of exon 4 and 39 bp of exon 5 of the bovine GHR gene, prepared in a previous study conducted in this laboratory (Wang et al., 2003). The cDNA plasmid for bovine GHR 1A mRNA probe was a pGEM-T Easy plasmid containing a 312-bp cDNA insert that represented 191 bp of exon 1A, 81 bp of exon 2, and 40 bp of exon 3 of the bovine GHR gene (Kobayashi et al., 1999). The plasmids used to synthesize the bovine IGFBP-3 and ALS mRNA probes were pGEM-T Easy-based plasmids containing a 232-bp bovine IGFBP-3 and a 197-bp bovine ALS cDNA inserts, respectively

(Jiang, unpublished data). The plasmid for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a pGEM-T Easy plasmid containing a 90-bp bovine GAPDH cDNA (Wang et al., 2003).

The radioactive antisense probes for RPA were synthesized by using α-^{32}P-CTP and the Riboprobe Combination Systems kit (Promega, Madison, WI). Briefly, in vitro transcription was performed in a 25 μL of solution (5 μL of 5 × transcription buffer, 2.5 μL of 0.1 *M* DTT, 1 μL of 10 m*M* ATP, 1 μL of 10 m*M* GTP, 1 μL of 10 m*M* UTP, 1 μL of 100 μM CTP, 1 μL of Rnase inhibitor, 0.5 μg of linearized plasmid DNA template, 5 μL of α-^{32}P-CTP and 1 μL of RNA polymerase), and incubated at 37 °C for 1 h. Following the incubation, 2 μL of Rnase-free Dnase I was added to the solution, and then the solution was incubated at 37 °C for 20 min. Following the digestion, free α-^{32}P-CTP was removed by phenol-chloroform extraction, and the probe was purified by filtrating the solution through Quick Spin Sephadex G-50 columns (Roche Molecular Biochemicals, Indianapolis, IN). The radioactivity of the purified probe was determined by liquid scintillation counting in a Backman LS 5000 TA (Beckman Instruments Inc., Fullerton, CA).

RPA was carried out by using the RPA II kit (Ambion, Austin, TX). Twenty micrograms of total RNA each sample were used in the RPA reaction for detecting liver IGF-I, total GHR, GHR 1A and non-GHR 1A, skeletal muscle and adipose IGF-I mRNA. Fifteen micrograms of total RNA from each sample were used in the RPA reaction of liver IGFBP-3 and ALS mRNA. Each hybridization tube contained ~8 × 10^5 dpm of probe for IGF-I, GHR, GHR 1A, ALS or IGFBP-3 mRNA, and ~2 × 10^5 dpm of probe for GAPDH mRNA. The hybridization was incubated at 42 °C for 16 h. Following the hybridization, non-hybridized RNA fragments were digested by Rnase A/T1 at 37 °C for 40 min. After digestion, the ribonuclease-protected RNA

fragments were resolved by electrophoresis on 6% acrylamide gels containing 7 *M* urea. After gel electrophoresis, the radioactivity of ribonuclease-protected bands was detected by exposure to phosphor screens and quantified by using the ImageJ program as described above. The density of the protected IGF-I, totGHR, GHR 1A, non-GHR 1A, IGFBP-3 or ALS mRNA band was normalized to that of the protected GAPDH mRNA band in the same RNA sample, and the normalized density was used to represent the abundance of the corresponding mRNA.

Western ligand blot assay

This assay was used to compare the abundance of serum IGFBP-3 protein between food-deprived and fed steers. Four microliters of serum were heated at 60 °C for 10 min in non-reducing Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), and then resolved by electrophoresis on 12% SDS-polyacrylamide gels at 150 volt for 3 h. The proteins in gels were electro-transferred to nitrocellulose membranes by running at 55 V for 2 h. The membrane was washed with 3% igepal CA-630 in Tris buffered saline (TBS, pH 7.4) for 30 min, and blocked with 1% BSA in TBS (pH 7.4) for 2 h. After being washed with 0.1% Tween-20 in TBS (pH 7.4) for three times, the membrane was incubated with 1.25×10^6 dpm/mL of ^{125}I-labeled IGF-I at 4 °C overnight. After being washed with 0.1% Tween-20 in TBS (pH 7.4) for three times and TBS (pH 7.4) for two times, the membrane was dried at 37 °C, and then exposed to a X-ray film for 4 d at –80 °C. The densities of the bands in the image were quantified by using the ImageJ program as described above.

Liver whole cell lysates and microsomal membrane extractions

For preparation of liver whole cell lysates, 0.5 g of frozen liver tissue was homogenized in 2 mL of ice-cold lysis buffer A (10 m*M* Tris, 1 m*M* EGTA, 1 m*M* EDTA, 150 m*M* NaCl, and 1% Triton X-100, pH 7.6) containing protease inhibitors (1 m*M* Na_3VO_4, 1 m*M* Na

pyrophosphate, 10 m*M* NaF, 1 m*M* PMSF, 10 mg/L aprotinin and 10 mg/L Leupeptin) at 20,000 rpm for 1 min. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C, and the supernatant were centrifuged again at 12,000 × g for 10 min at 4 °C. The final supernatant were collected and stored at –80 °C.

For extraction of liver microsomal membrane proteins, 0.5 g of frozen liver tissue was homogenized in 2.5 mL of ice-cold sucrose buffer (50 m*M* Tris, 250 m*M* sucrose, 5 m*M* EGTA, and 150 m*M* NaCl, pH 7.6) containing protease inhibitors (described above), at 20,000 rpm for 1 min. The homogenate was centrifuged at 11,000 × g for 20 min at 4 °C. The supernatant were collected and centrifuged again at 100,000 × g for 1 h at 4 °C. The pellets were re-suspended in 1 mL of lysis buffer A, and the solution was centrifuged again at 105,000 × g for 1 h at 4 °C. The pellets were dissolved in 0.5 mL of ice-cold lysis buffer A, and the microsomal membrane extractions were stored at – 80 °C for further assays. Protein concentration of each sample was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), following the manufacturer's instructions.

Western blot analysis

Liver whole cell lysates (40 μg of protein) or microsomal membrane proteins (20 μg of protein) respectively were resolved by electrophoresis on SDS-polyacrylamide gel (8% for detection of GHR, JAK2 and STAT5; 10% for ALS), and then electro-transferred to nitrocellulose membranes. After being blocked with 5% milk in TBST (Tris buffered saline contained 0.1% Tween-20) for 1 h at room temperature, the nitrocellulose membrane was incubated with a primary antibody at 4 °C overnight. After being washed with TBST for three times, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit or donkey anti-mouse IgG-HRP antibody, Santa Cruz

biotechnology, Santa Cruz, CA) at 1: 2000 dilutions for 2 h at room temperature. After three times of washing with TBST, the membrane was incubated in SuperSignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL) for 5 min, and the chemiluminescence signal was detected with X-ray films and quantified using the ImageJ program as described above.

For the detection of GHR, the primary antibody was anti-$GHR_{cyt\text{-}AL47}$ used at the dilution of 1:1000, which was a rabbit antiserum raised against a bacterially expressed N-terminally His-tagged fusion protein incorporating human GHR residues 271-620 (Zhang et al., 2001b). The primary antibody for detection of JAK2 was anti-JAK_{AL33} at the dilution of 1:1000, a rabbit polyclonal anti-sera directed at residues 746-1129 of murine JAK2 (Jiang et al., 1998). The primary antibody for detection of STAT5 was anti-STAT5 at the dilution of 1:1000, a rabbit polyclonal affinity-purified antibody raised against a peptide mapping at the C-terminus of STAT5 of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody for detection of ALS was anti-ALS 1082 antibody at the dilution of 1:3000 for liver ALS and 1:5000 for serum ALS, a rabbit antiserum raised against a KLH-conjugated peptide with 28 residues of bovine ALS mapping at the carboxyl terminal (Kim et al., 2006). The endogenous control β-actin was detected by an antibody from Cell Signaling Technology (Danvers, MA) at the dilution of 1:500.

For the detection of liver hosphor-JAK2 (pJAK2) and hosphor-STAT5 (pSTAT5) proteins, liver whole cell lysates containing 80 μg of protein were resolved by electrophoresis on 8% polyacrylamide gels. Anti-phospho-JAK2 (Upstate USA, Lake Placid, NY) was raised against KLH-conjugated, synthetic peptide corresponding to amino acids surrounding hosphor-tyrosines 1007 and 1008 of human JAK2, and was used at 1:500 dilution.

Anti-phospho-STAT5A/B (diluted by 1:500) was a mouse monoclonal antibody raised against KLH-conjugated, synthetic hosphor-peptide (KAVDG[pY]VKPQIK) corresponding to amino acids 689-700 of STAT5A and 694-705 of STAT5B (Upstate USA, Inc., Lake Placid, NY). After being stripped in Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL), the membranes were redetected with Anti-JAK_{AL33} (diluted at 1:1000) and Anti-STAT5 (diluted at 1:1000), respectively. In the pSTAT5 analysis, 30 μg of whole cell lysates from CHO cells that were transfected with a STAT5 expression construct and a GHR expression construct (Wang and Jiang, 2005), and treated with growth hormone for 30 min, was used as a positive control, and CHO cells that were not transfected and not treated with growth hormone as a negative control.

For the detection of serum ALS, serum (0.5 μL) from each steer was resolved by 10% SDS-PAGE, then transferred to nitrocellulose membrane, and blotted with the anti-ALS 1082 antibody as described above.

Reverse transcription and real time-PCR (RT-PCR)

Reverse transcription coupled with real time quantitative PCR was used to quantify the abundance of liver SOCS2 and CIS mRNAs, and muscle IGF-I mRNA. Total RNA were reverse-transcribed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Briefly, 0.4 μg of total RNA was reverse-transcribed in 20 μL containing 110 nmol $MgCl_2$, 10 nmol of dNTPs, 8 μU of ribonuclease inhibitor, 50 pmole of random hexamers, 0.50 μL of 50 U /μL multiscribe reverse transcriptase, and 2 μL of 10 × reverse transcription buffer. The reverse transcription reaction was performed at 25 ºC for 10 min, 48 ºC for 30 min, and 95 °C for 5 min.

The PCR was set up by using a Power Syber Green PCR Master Mix kit (Applied Biosystems, Foster City, CA). The total volume of PCR reaction was 25 μL, containing 2 μL of reverse-transcribed products that corresponded to 0.02 μg of liver total RNA or 6 μL corresponded to 0.06 μg of total muscle RNA, 12.5 μL of Syber Green PCR Master Mix, 0.5 μL of 10 μM forward primer and 0.5 μL of 10 μM reverse primer (Table 3-1). The PCR were run on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative abundance of SOCS2, CIS or IGF-I mRNA was calculated using the $2^{-\Delta Ct}$ method, according to the manufacturer's directions. Ct is threshold cycle representing the fractional cycle number at which the fluorescence passes the threshold. ΔCt is the normalization of SOCS2, CIS or IGF-I mRNA Ct to endogenous control GAPDH mRNA Ct: ΔCt = Ct for target mRNA – Ct for GAPDH mRNA.

Statistical analysis

The effect of treatment (fed versus food-deprived) was tested using the PROC GLM procedure of SAS 9.0 (SAS Institute, Inc., Cary, NC). Dependent variables were serum concentration of IGF-I, IGFBP-3 and ALS, liver GHR, IGF-I, IGFBP-3, ALS, SOCS2 and CIS mRNAs, muscle and adipose IGF-I mRNAs, liver GHR, STAT5, JAK2 and ALS proteins, and IGF-I stability. The following model was used: $Y_{ij} = \mu + \alpha_i + E_{ij}$. Y_{ij} represents dependent variable corresponding with the ith treatment of the jth steer. The mean is μ, α_i is the fixed effect of the ith treatment, and E_{ij} is the residual error. Results are presented as LS mean ± SE.

RESULTS

Food deprivation decreased serum IGF-I concentration

In the fed steers, serum IGF-I concentration was 183.6 ± 12.8 ng/mL. In the food-deprived steers, it was 97.8 ± 10.7 ng/mL (Figure 3-1). Thus, 60-h food deprivation caused a 46.8% decrease in serum IGF-I concentration ($P < 0.01$).

^{125}I-labeled IGF-I was as stable in food-deprived steer serum as in fed steer serum

Because serum IGF-I concentration was decreased in food-deprived steers, we tested the hypothesis that the decrease was due to increased serum IGF-I degradation during food deprivation. ^{125}I-labeled IGF-I was incubated with sera from fed and food-deprived steers at 37 °C for 45 h, and the undegraded ^{125}I-IGF-I was resolved by 8% SDS-PAGE. As shown in Figure 3-2A and 3-2B, there was no difference in the abundance of undegraded ^{125}I-IGF-I between fed and food-deprived sera after 45 h of incubation ($P = 0.73$). Indeed, little of ^{125}I-IGF-I was degraded in sera within 46 h of incubation, while more than a half of it was degraded after 6 h of incubation with different proteases (Figure 3-2C).

Liver IGF-I mRNA was lower in food-deprived steers than fed steers

Since the decrease in serum IGF-I in food-deprived steers did not appear to be due to IGF-I degradation in serum, we determined whether the IGF-I synthesis in liver was decreased by food deprivation. RPA was used to quantify total IGF-I mRNA (Figure 3-3A). Liver IGF-I mRNA level was 82.4% lower in food-deprived steers than in fed steers ($P < 0.01$, Figure 3-3B), which indicated that IGF-I gene transcription or IGF-I mRNA stability or both in liver were decreased by food deprivation. There was a highly positive correlation between liver IGF-I mRNA abundance and serum IGF-I concentration ($r = 0.92$, $P < 0.01$), further suggesting that the

decrease in liver IGF-I mRNA expression contributed to the decrease in serum IGF-I level during food deprivation.

Subcutaneous adipose IGF-I mRNA was lower in food-deprived steers than in fed steers

Decreased IGF-I gene expression in non-hepatic tissues might also contribute to the decrease in serum IGF-I during food deprivation. For this reason, subcutaneous adipose and skeletal muscle IGF-I mRNAs were quantified by RPA (Figure 3-4A and C). Subcutaneous adipose IGF-I mRNA tended to be lower in food-deprived steers than in fed steers ($P = 0.058$, Figure 3-4B). Skeletal muscle IGF-I mRNA was barely detectable by RPA (Figure 3-4C). Based on a real time-PCR, skeletal muscle IGF-I mRNA was not different between fed and food-deprived steers ($P = 0.18$, Figure 3-4D).

Decreased liver IGF-I mRNA by food deprivation was associated with decreased liver total GHR mRNA

Liver IGF-I biosynthesis is mainly under the control of GH, which acts through binding to GHR located in the cell membrane. Therefore, one possible reason for decreased liver IGF-I mRNA expression during food deprivation might be decreased expression of liver GHR. As measured by RPA (Figure 3-3A), liver total GHR mRNA was 47.62% lower in food-deprived steers than in fed steers ($P < 0.01$; Figure 3-3B). A Pearson correlation analysis indicated that liver total GHR mRNA was strongly associated with liver IGF-I mRNA ($r = 0.94$, $P < 0.01$).

The decrease in liver total GHR mRNA by food deprivation was mainly due to the decrease in GHR 1A mRNA

The GHR 1A mRNA is the major GHR mRNA variant in the liver, and is exclusively expressed in the liver (Jiang and Lucy, 2001). An RPA was carried out to determine the difference in liver GHR 1A mRNA between fed and food-deprived steers. The probe for GHR

1A mRNA contained part of exon 1A, exon 2 and part of exon 3 of the GHR gene, so it detected not only the GHR 1A mRNA but also other liver GHR variants (non-GHR 1A) due to the hybridization of the probe with exons 2 and 3 of the GHR gene (Figure 3-5A). The RPA indicated that liver GHR 1A mRNA in food-deprived steers was 65.5% lower than in fed steers ($P < 0.01$), and that non-GHR 1A was 19.5% lower in food-deprived steers than in fed steers ($P < 0.05$). When GHR 1A and non-GHR 1A mRNA were combined as total GHR mRNA, it was found that liver total GHR mRNA was 48.3% lower in food-deprived steers than in fed steers ($P < 0.01$) (Figure 3-5B). In fed steers, GHR 1A mRNA accounted for 62.7% of liver total GHR mRNA, whereas in food-deprived steers, it represented only 41.8% of liver total GHR mRNA. These data indicated that the decrease in liver total GHR mRNA in food-deprived steers was mainly due to the decrease in GHR 1A mRNA ($r = 0.98$, $P < 0.01$).

Liver GHR protein was lower in food-deprived steers than in fed steers

Since liver GHR mRNA was significantly lower in food-deprived steers than in fed steers, Western blot was used to determine whether liver GHR protein level was also lower in food-deprived steers (Figure 3-6A). The analysis detected two GHR protein bands: precursor GHR (pGHR) and mature GHR (mGHR) that is glycosylated compared to pGHR (Harding et al., 1994). Compared to the fed steers, food deprived steers had 19.0% less mGHR ($P < 0.05$), 21.9% less pGHR ($P = 0.073$) and 20.4% less total GHR ($P < 0.05$) in liver whole cell lysates (Figure 3-6B). A Pearson correlation analysis indicated that liver GHR 1A mRNA was strongly associated with liver total GHR protein ($r = 0.78$, $P < 0.05$).

Compared to the fed steers, food-deprived steers had 58.5% lower mGHR protein ($P < 0.01$), 45.2% lower pGHR ($P < 0.05$) and 52.0% lower total GHR ($P < 0.01$) in liver microsomal membrane extracts (Figure 3-7A and B). The decrease in microsomal membrane GHR protein (–

52.0%) was greater than that in liver whole cell lysates (– 20.4%), suggesting that food deprivation further decreased the proportion of microsomal membrane GHR protein in addition to decreasing GHR protein synthesis. There was also a positive correlation between microsomal mGHR protein and liver IGF-I mRNA ($r = 0.90$, $P < 0.01$).

Food deprivation did not change liver STAT5 and hosphor-STAT5, JAK2 and phospho-JAK2 protein levels

Based on Western blot analyses, there were no differences in the abundance of liver STAT5 ($P = 0.55$, Figure 3-8A and C), pSTAT5 ($P = 0.81$, Figure 3-8B and C), JAK2 ($P = 0.53$, Figure 3-9A and C) or pJAK2 ($P = 0.70$, Figure 3-9B and C) proteins between fed and food-deprived steers. Therefore, food deprivation do not alter the amount of total and active proteins of these signaling molecules, indicating that the decrease in liver IGF-I mRNA abundance by food deprivation may be due to other GHR signaling pathways other than JAK2/STAT5 pathway, decreased mRNA stability, or both.

Food deprivation increased liver SOCS2 and CIS mRNA levels

Real time-PCR was used to quantify the abundance of SOCS2 and CIS mRNA in fed and food-deprived steer livers. As shown in Figure 3-10, food deprivation increased liver SOCS2 mRNA level by 126.6% ($P < 0.01$), and liver CIS mRNA by 56.8% ($P < 0.05$). Negative correlations were found in liver IGF-I mRNA with SOCS2 ($r = -0.67$, $P < 0.05$) and CIS ($r = -0.70$, $P < 0.05$). These results indicate that these negative regulatory proteins may be involved in down-regulation of liver IGF-I mRNA expression under food deprivation.

Food deprivation decreased serum IGFBP-3 concentration and liver IGFBP-3 mRNA

Western ligand blotting was used to detect serum IGFBP-3 level in steers (Figure 3-11A). The assay indicated that food-deprived steers had 38.7% lower IGFBP-3 ($P < 0.05$) than fed steers in the blood (Figure 3-11B).

To determine whether the decrease in serum IGFBP-3 was due to decreased expression of IGFBP-3 mRNA in the liver, liver IGFBP-3 mRNA abundance was measured by RPA (Figure 3-12A). The data indicated liver IGFBP-3 mRNA was 59.0% lower in food-deprived steers ($P < 0.01$) than in fed steers (Figure 3-12B).

Food deprivation did not alter serum and liver ALS protein abundance, but decreased liver ALS mRNA level

Western blot was used to detect serum and liver ALS levels (Figure 3-13A and B). Both serum and liver ALS abundance were not different between fed and food-deprived steers ($P = 0.70$ and 0.34, respectively; Figure 3-13C). However, liver ALS mRNA was 63.6% lower in food-deprived steers than in fed steers ($P < 0.01$; Figure 3-12A and B).

DISCUSSION

Serum IGF-I is decreased under food deprivation. This was observed by many others (Zhang et al., 1998; Lee et al., 2002; Wang et al., 2003) and was confirmed in our study: serum IGF-I concentrations of food-deprived steers were only 54% of those of fed steers. An obvious possible mechanism by which serum IGF-I is decreased during food deprivation is that serum IGF-I degradation is increased. Surprisingly, this possibility had not been tested. In this study, we compared the stability of ^{125}I-IGF-I in the sera of fed and food-deprived steers, and found no differences between them. This observation suggests that decreased serum IGF-I under food deprivation is not due to increased degradation of serum IGF-I. We also unexpectedly noticed that ^{125}I-labeled IGF-I was very stable in bovine serum *in vitro*. It appears that degradation is not a major way by which blood IGF-I concentration is decreased in animals.

Two other possible reasons for serum IGF-I being decreased under food deprivation are that tissue production of IGF-I is decreased and that clearance of IGF-I form blood is increased. Several studies indicated food deprivation or food restriction decreased liver IGF-I mRNA (Kirby et al., 1993; Zhang et al., 1998; Wang et al., 2003). Consistent with these reports, our study indicated that liver IGF-I mRNA was lessened by 82% after a 60-h food deprivation. We also observed that there was a highly positive correlation between serum IGF-I and liver IGF-I mRNA level. These observations together with the fact that 75% of circulating IGF-I comes from liver (Sjogren et al., 1999) suggest that decreased liver IGF-I gene expression contributes at least in part to the decrease in serum IGF-I under food deprivation. Besides liver, IGF-I from other peripheral tissues contribute to ~ 25% of circulating IGF-I (Sjogren et al., 1999). It was found that IGF-I mRNA in subcutaneous adipose was slightly decreased in food-deprived steers,

suggesting that the decrease in circulating IGF-I during food-deprivation may be also partly due to decreased IGF-I expression in adipose tissue.

Liver IGF-I synthesis is dependent on growth hormone, which acts by binding to growth hormone receptor (Mathews et al., 1986; Shoba et al., 1999; Coschigano et al., 2003). Growth hormone receptor is expressed in many tissues, with the highest level in liver (Tiong and Herington, 1991; Jiang and Lucy, 2001). Our study showed liver GHR expression under food deprivation was diminished at both mRNA and protein levels. We further found that the decrease in liver GHR mRNA was mainly due to decreased expression of GHR 1A mRNA variant. These results are consistent with an earlier report from this laboratory (Wang et al., 2003). Food deprivation caused a 20% decrease in liver total GHR protein, but a 52% decrease in liver microsomal membrane GHR. These results indicate that not only GHR protein synthesis but also membrane insertion of GHR may be down-regulated by food deprivation. There was a highly positive correlation between liver IGF-I RNA expression and GHR 1A mRNA and microsomal membrane mature GHR ($P < 0.01$). So food deprivation may reduce the availability of membrane GHR, thereby contributing to decreased IGF-I synthesis in the liver.

The JAK2-STAT5 pathway is the major GHR signaling pathway that mediates GH-induced IGF-I production (Davey et al., 2001; Rawlings et al., 2004). Decreased IGF-I mRNA expression could therefore be due to reduced activation of this pathway in the liver during food deprivation. Surprisingly, we observed that phospho-STAT5 protein or phospho-JAK2 protein in liver whole cell lysates was not altered by food deprivation. This observation suggests that decreased IGF-I mRNA expression in the liver during food deprivation is not due to decreased JAK2/STAT5 signaling from GH receptor.

Gene knockout mice and cell transfection studies indicate both SOCS2 and CIS are negative regulatory proteins of GHR signaling pathway (Matsumoto et al., 1997; Metcalf et al., 2000). Our study showed both liver SOCS2 and CIS mRNA expressions were increased in food-deprived steers, suggesting decreased IGF-I mRNA expression in the liver may be due to increased down-regulation of the GHR signaling pathway by SOCS2 and CIS during food deprivation. Food deprivation did not appear to down-regulate pSTAT5 or pJAK2, so how increased SOCS2 and CIS attenuate GHR signaling transduction to IGF-I gene expression remains an interesting question to be addressed in the future.

In normal physiological status, the majority of IGF-I circulates as a ternary complex with IGFBP -3 and ALS, which decrease the clearance of IGF-I from blood (Binoux and Hossenlopp, 1988; Albiston and Herington, 1990; Yakar et al., 2002b; Nindl et al., 2003). We observed that, although serum ALS was not different after 60-h food deprivation, serum IGFBP-3 in steers was decreased. This result indicated that the clearance of IGF-I from blood may be increased in food-deprived steers. Decreased serum IGFBP-3 was associated with decreased liver IGFBP-3 mRNA, suggesting that decreased liver IGFBP-3 expression contributes to the decrease in serum IGFBP-3 during food deprivation. Paradoxically, while liver ALS mRNA was decreased, both serum and liver ALS proteins were not decreased after a 60-h food deprivation. There results seem to indicate that translational efficiency of liver ALS is increased during food deprivation.

In summary, food deprivation decreases serum IGF-I concentration in steers. This decrease was not due to increased degradation of IGF-I in serum. Food deprivation also decreased liver IGF-I mRNA level, and this decrease was highly correlated with the decrease in serum IGF-I. The decrease in liver IGF-I mRNA was associated with decreased expression of liver GHR 1A mRNA and microsomal membrane GHR protein. Food deprivation increased the

expression of GHR signaling negative regulatory factors SOCS2 and CIS mRNAs in the liver. These increases may contribute to the decreased IGF-I production in the liver during food deprivation. Food deprivation also decreased subcutaneous adipose IGF-I mRNA, and this decrease may contribute to the decrease in serum IGF-I too. Food deprivation decreased liver IGFBP-3 mRNA and serum IGFBP-3 protein, which may lead to increased clearance of IGF-I from blood. Therefore, multiple mechanisms seem to mediate the decrease in serum IGF-I concentration in steers during food deprivation.

Table 3-1. Primers used in real-time PCR

mRNA	GenBank #	Name	Primer sequence (from 5' to 3')	Product length
SOCS2	BC114662	bSOCS2F1	TCGCATCGAATACCAAGATG	129 bp
		bSOCS2R1	GTCCGCTTATCCTTGCACAT	
CIS	BC113307	bCISH2F1	TTCCTGGAGGAGGCAGTAGA	113 bp
		bCISH2R1	TCCCGAAGGTAGGAGAAGGT	
GAPDH	NM_002046	GAPDH-F	ACCATCTTCCAGGAGCGAGA	338 bp
		GAPDH-R	GTCTTCTGGGTGGCAGTGAT	

Figure 3-1. Food deprivation decreased serum IGF-I concentration.

Serum IGF-I concentrations (ng/mL) of 10 steers were determined by radioimmunoassay. Each bar is mean ± pooled SE (n = 5). ** P < 0.01 versus Fed.

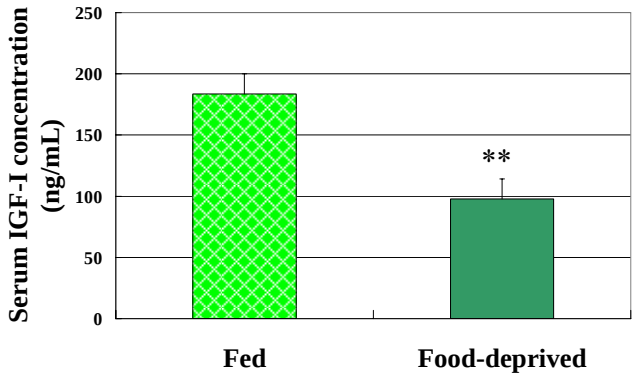
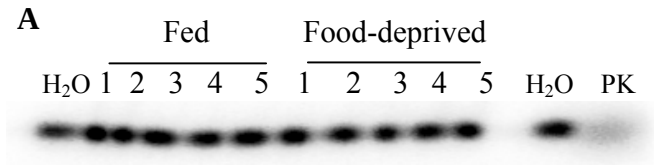
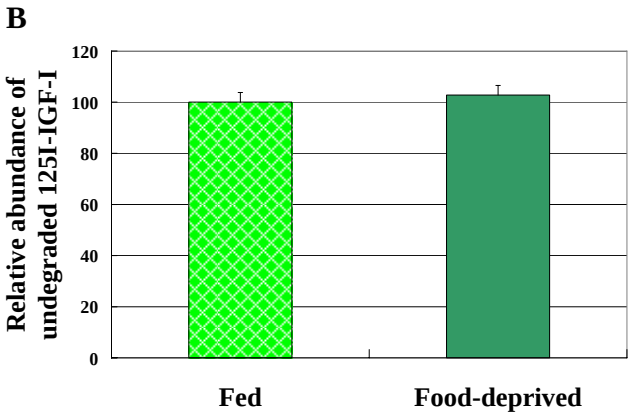


Figure 3-2. Comparison of ^{125}I-IGF-I stability in the sera of fed and food-deprived steers.

A: ^{125}I-IGF-I was incubated at 37 °C in serum or H_2O (negative control) for 45 h, or in Proteinase K (PK) (positive control) for 6 h, followed by gel electrophoresis. **B:** Densitometric quantification of undegraded ^{125}I-IGF-I in panel A. Each bar is mean ± pooled SE (n = 5). P = 0.74 Fed versus Food-deprived. **C:** Gel electrophoresis of ^{125}I-IGF-I incubated at 37 °C in H_2O, Proteinase K (PK), Collagenase type 2 (CO), Trypsin EDTA (TS) and serum (SR) for different hours (h).





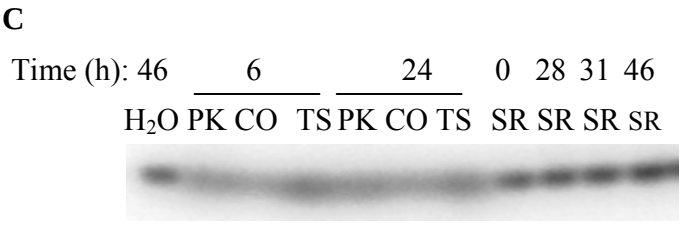


Figure 3-3. Liver total IGF-I and total GHR mRNA abundance in the fed and food-deprived steers.

A: RPA image of liver total GHR (totGHR), IGF-I and GAPDH (loading control) mRNAs. The ribonuclease-protected RNA fragments were indicated. P: probes. Y: yeast RNA as negative control. **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of IGF-I or total GHR bands was normalized to that of GAPDH band of the same animal. There was no difference in the abundance of GAPDH mRNA between fed and food-deprived steers (P = 0.42). Each bar is mean ± pooled SE (n = 5). ** P < 0.01 versus Fed.

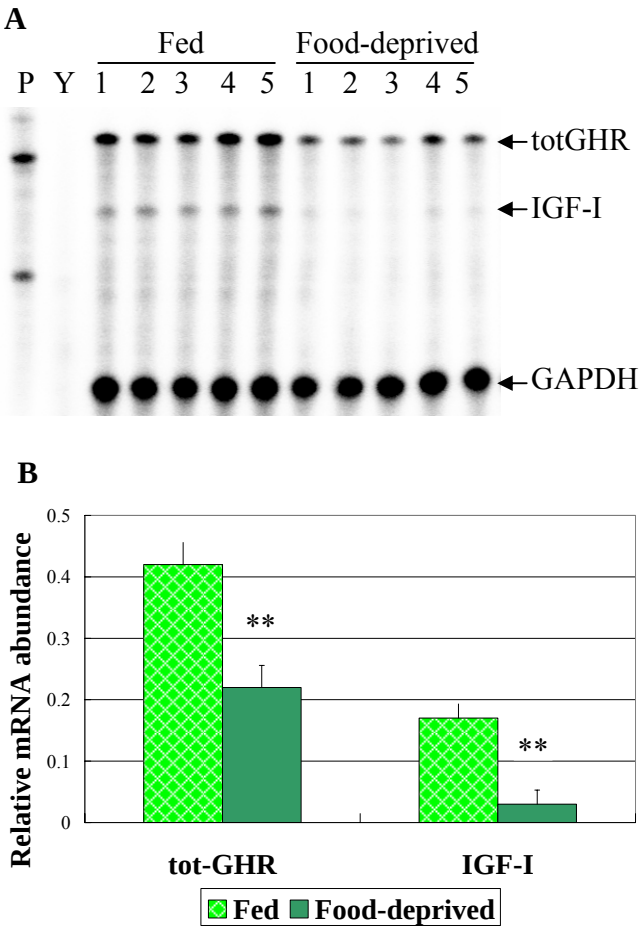
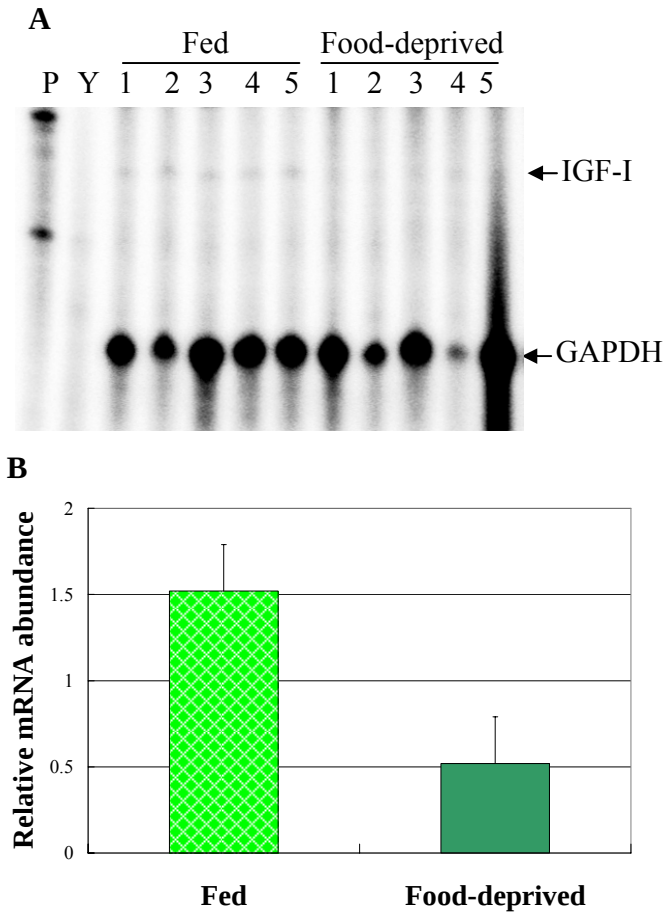
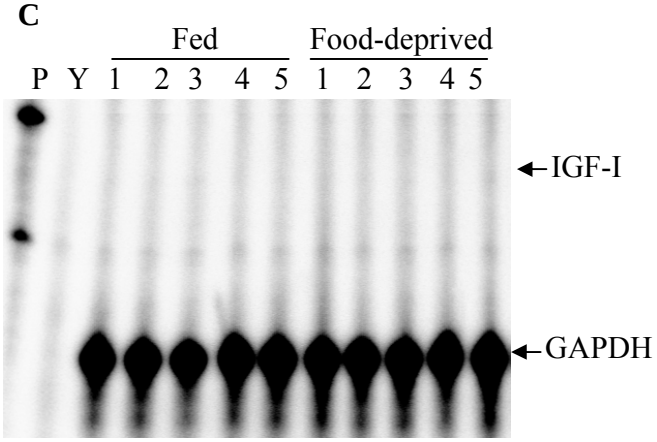


Figure 3-4. Subcutaneous adipose and skeletal muscle total IGF-I mRNA levels in the fed and food-deprived steers.

A: RPA image of subcutaneous adipose total IGF-I and GAPDH mRNAs. The ribonuclease-protected RNA fragments were indicated. P: probes. Y: yeast RNA (negative control). **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of a IGF-I band was normalized to that of GAPDH band of the same animal. There was no difference in the abundance of GAPDH mRNA between fed and food-deprived steers (P = 0.40). Each bar is mean ± pooled SE; P = 0.058, Fed versus Food-deprived. **C:** RPA image of skeletal muscle total IGF-I and loading control GAPDH mRNAs. **D:** Real time-PCR of skeletal muscle total IGF-I mRNA. Each bar is mean ± pooled SE, P = 0.18 Fed versus Food-deprived.



C


Fed
Food-deprived
P Y 1 2 3 4 5 1 2 3 4 5
IGF-I
GAPDH

D

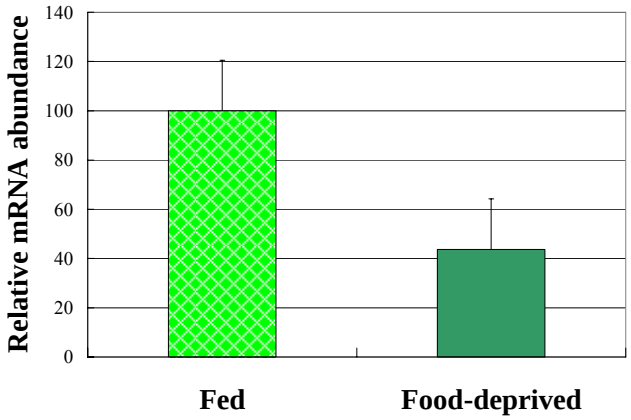

Relative mRNA abundance
140
120
100
80
60
40
20
0
Fed
Food-deprived

Figure 3-5. Liver GHR 1A and non-1A GHR mRNA abundance in the fed and food-deprived steers.

A: RPA image of liver GHR 1A, non-1A GHR and loading control GAPDH mRNAs. The ribonuclease-protected RNA fragments were indicated. P: probes. Y: yeast RNA as negative control. **B:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of GHR 1A, non-1A GHR mRNA bands were normalized to that of GAPDH band of the same animal. The combined abundance of GHR 1A and non-1A mRNA is used to represent total GHR (totGHR) mRNA abundance. There was no difference in the abundance of GAPDH mRNA between fed and food-deprived steers (P = 0.45). Each bar is mean ± pooled SE. * P < 0.05, and ** P < 0.01 versus Fed.

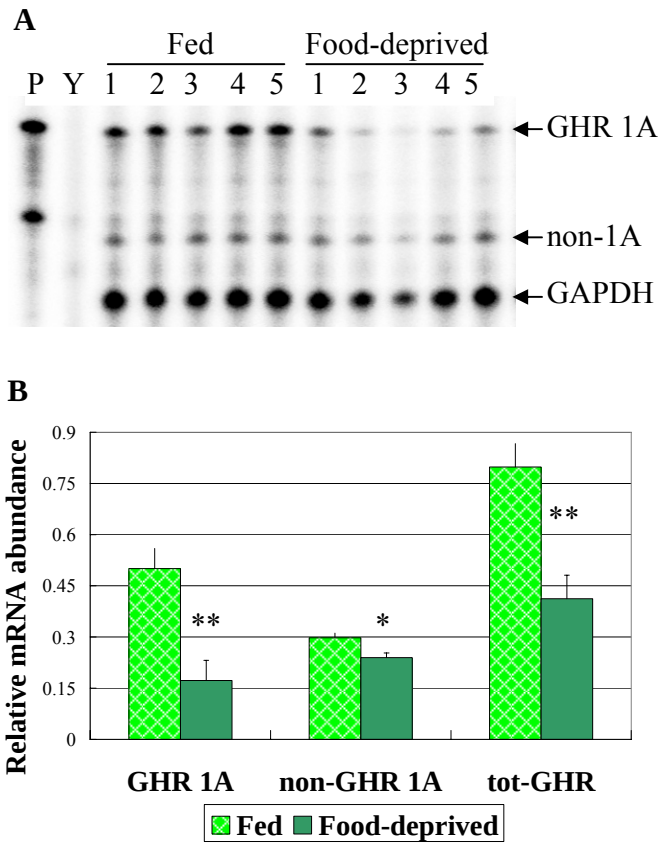


Figure 3-6. GHR protein levels in liver whole cell lysates of fed and food-deprived steers.

A: Images of Western blot analyses of liver whole cell lysates. mGHR: mature GHR; pGHR: precursor GHR; totGHR: total GHR; FD: food-deprived steer; F: fed steer. **B:** Densitometric quantification of bands in panel A. The abundance of GHR protein in food-deprived steers was expressed as percentage of that in fed steers. There was no difference in the abundance of β-actin between fed and food-deprived steers (P = 0.51). Each bar is mean ± pooled SE. * P < 0.05 versus Fed. pGHR: P = 0.073.

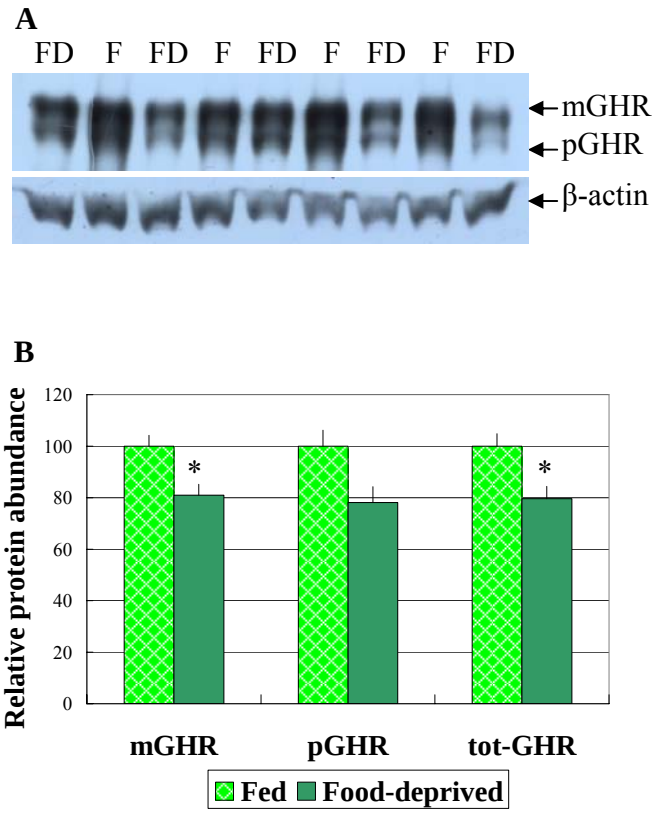


Figure 3-7. GHR protein levels in liver microsomal membrane extracts of fed and food-deprived steers.

A: Image of Western blot analysis of liver microsomal membrane extractions. mGHR: mature GHR; pGHR: precursor GHR; totGHR: total GHR; FD: food-deprived steer; F: fed steer. **B:** Image of a polyacrylamide gel that was stained with GelCode Blue Stain Reagent (PIERCE, Rockford, IL) after electro- transference to show equal protein loading and transfer. M: protein molecular marker. **C:** Densitometric quantification of bands in panel A. The abundance of GHR protein in food-deprived steers was expressed as percentage of that in fed steers. Each bar is mean ± pooled SE. * $P < 0.05$ and ** $P < 0.01$ versus Fed.

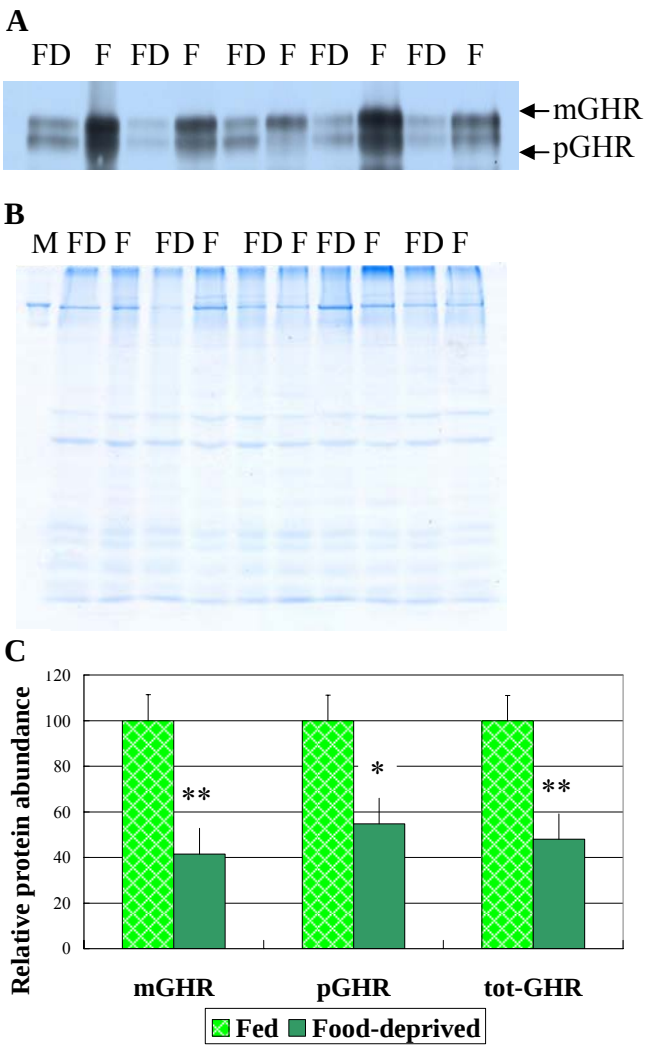


Figure 3-8. STAT5 and phospho-STAT5 protein abundance in liver whole cell lysates of fed and food-deprived steers.

A: Images of Western blot analyses of STAT5 and β-actin in liver whole cell lysates. FD: food-deprived steer; F: fed steer. **B:** Images of Western blot analyses of phospho-STAT5 (pSTAT5) in liver whole cell lysates. The membrane was stripped after the detection of pSTAT5, and reprobed with anti-STAT5 antibody. Positive control (PC) and negative control (NC) were lysates of CHO cells treated with or without GH respectively as described in Materials and Methods. **C:** Densitometric quantification of bands in panels A and B. The abundance of STAT5 or pSTAT5 protein in food-deprived steers was expressed as percentage of that in fed steers. Each bar is mean ± pooled SE. P = 0.55 for STAT5, and P = 0.82 for pSTAT5, Fed versus Food-deprived.

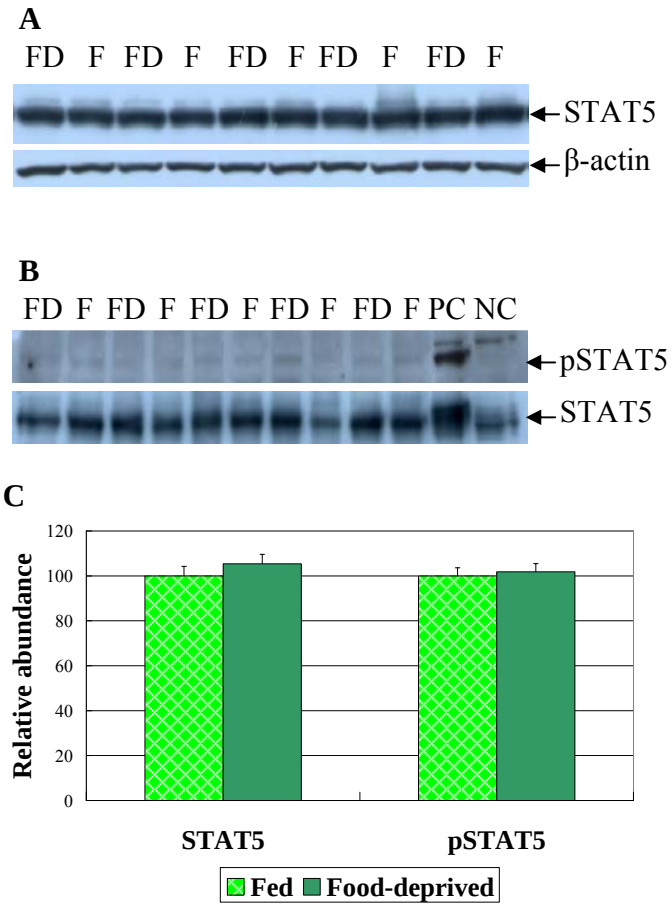


Figure 3-9. JAK2 and phospho-JAK2 abundance in liver whole cell lysates of fed and food-deprived steers.

A: Images of Western blot analyses of JAK2 and β-actin in liver whole cell lysates. FD: food-deprived steer; F: fed steer. **B:** Images of Western blot analyses of phospho-JAK2 (pJAK2) in liver whole cell lysates. The membrane was stripped after the detection of pJAK2, and then reprobed with anti-JAK2 antibody. **C:** Densitometric quantification of bands in panels A and B. The abundance of JAK2 or pJAK2 protein in food-deprived steers was expressed as percentage of that in fed steers. Each bar is mean ± pooled SE. P = 0.54 for JAK2, and P = 0.71 for pJAK2, Fed versus Food-deprived.

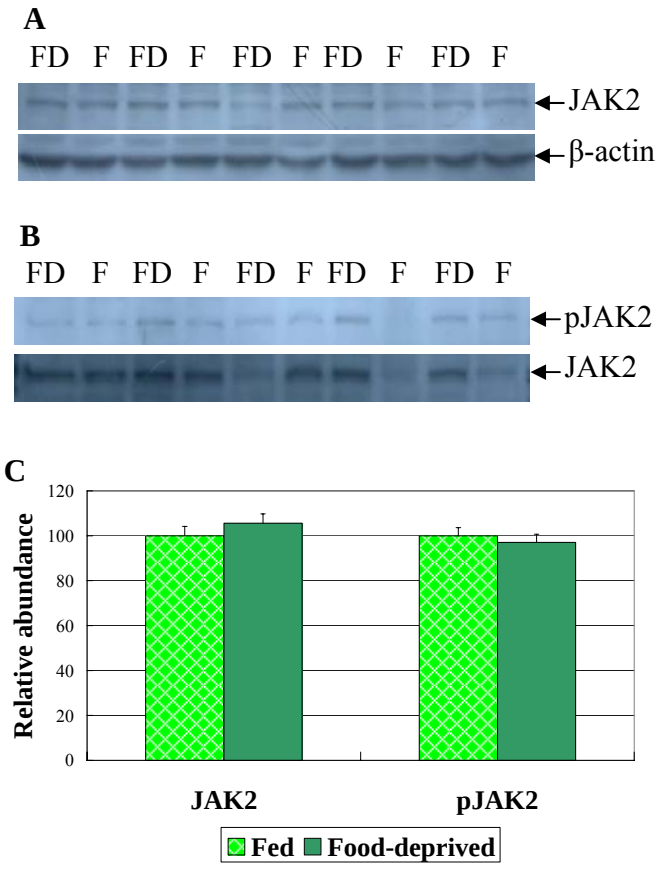


Figure 3-10. Food deprivation increased liver CIS and SOCS2 mRNA levels in steers.

A: CIS mRNA abundance quantified by real time-PCR. Each bar is mean ± pooled SE (n=5). * P < 0.05 versus Fed. **B**: SOCS2 mRNA abundance quantified by real time-PCR. Each bar is mean ± pooled SE (n=5). ** P < 0.01 versus Fed.

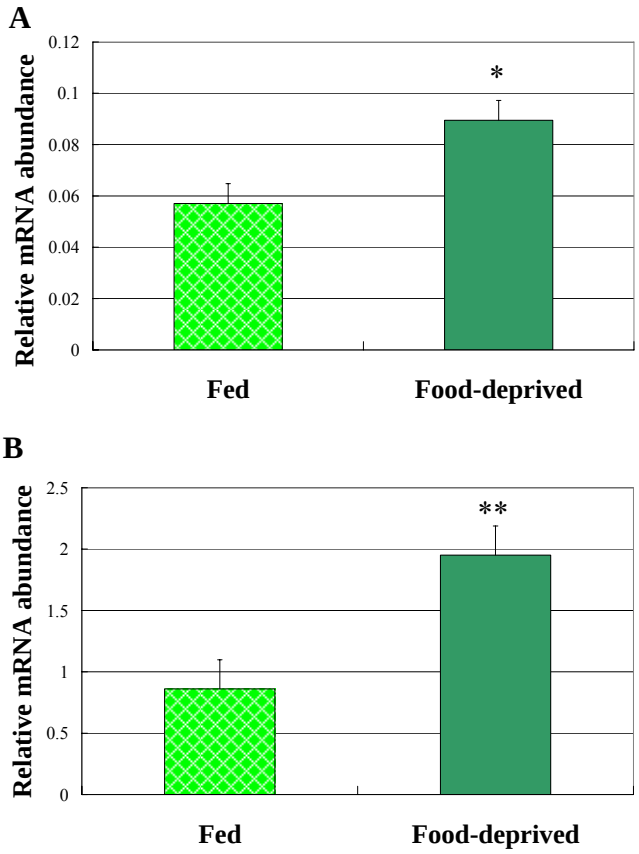


Figure 3-11. Serum IGFBP-3 levels in fed and food-deprived steers.

A: Image of Western ligand blotting analysis of serum IGFBP-3. **B:** Densitometric quantification of IGFBP-3 in panel A. The abundance of IGFBP-3 in food-deprived steers was expressed as percentage of that in fed steers. Each bar is mean ± pooled SE. * $P < 0.05$ versus Fed.

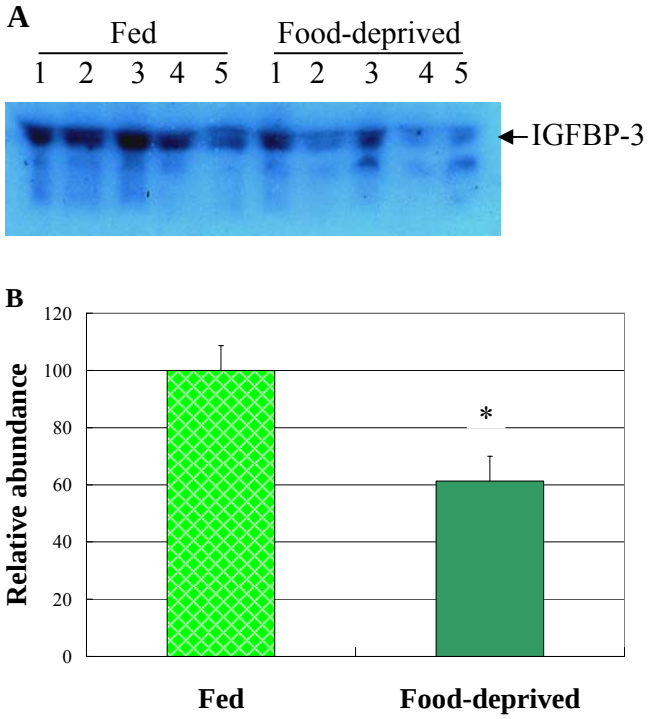


Figure 3-12. Liver IGFBP-3 and ALS mRNA abundance in the fed and food-deprived steers.

A: RPA image of liver IGFBP-3 and ALS and loading control GAPDH mRNAs. The ribonuclease-protected RNA fragments are indicated. P: probes. Y: yeast RNA (negative control).

B: Densitometric quantification of ribonuclease-protected mRNA bands in panel A. The density of an IGFBP-3 or ALS band was normalized to that of GAPDH band of the same animal. There was no difference in the abundance of GAPDH mRNA between fed and food-deprived steers (P = 0.50). Each bar is mean ± pooled SE. ** P < 0.01 versus Fed.

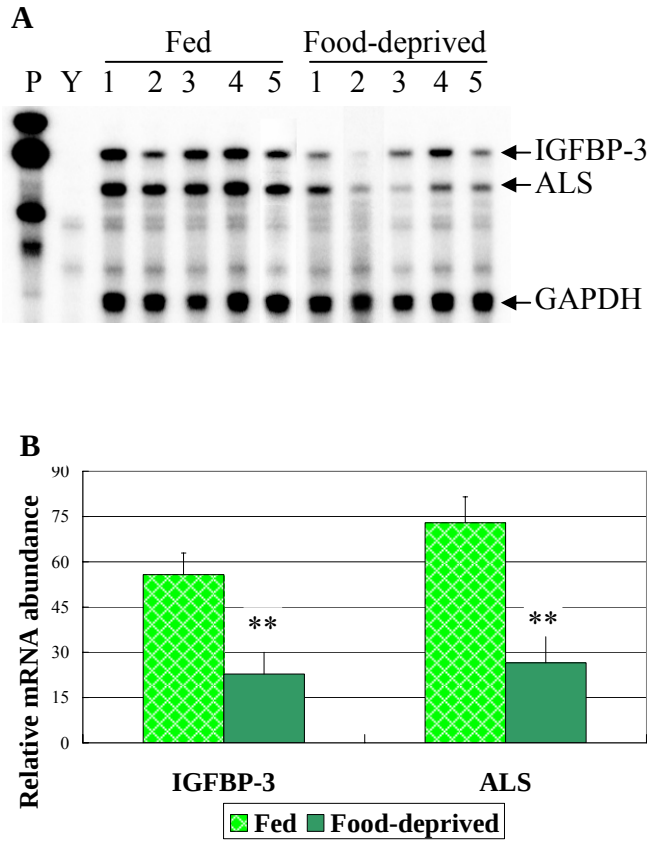
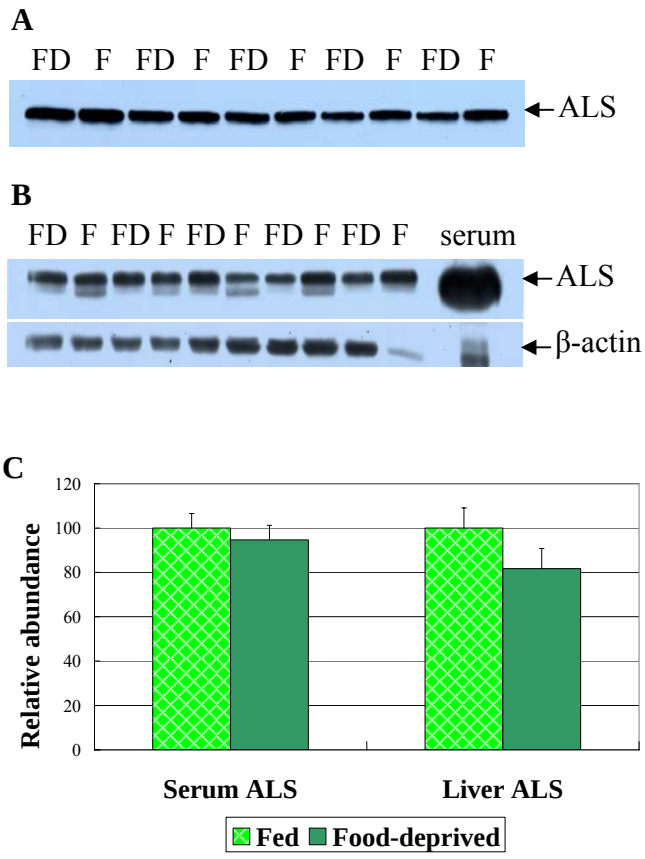


Figure 3-13. ALS protein levels in sera and liver whole cell lysates of fed and food-deprived steers.

A: Image of Western blot analysis of serum ALS. FD: food-deprived steer; F: fed steer. **B:** Images of Western blot analysis of ALS and β-actin in liver whole cell lysates. Serum (0.2 μL) was used as a positive control. **C:** Densitometric quantification of bands in panel A and B. The abundance of ALS protein in food-deprived steers was expressed as percentage of that in fed steers. There was no difference in the abundance of β-actin in liver whole cell lysates between fed and food-deprived steers (P = 0.79). Each bar is mean ± pooled SE. P = 0.70 for serum ALS protein; P = 0.34 for liver ALS protein.



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APPENDIX I

Statistical analysis for chapter II

1. Option for determining the denominator degrees of freedom (ddfm) for F-tests

Since data from the cow study were repeated measurements, Kenward-Rogers (kr) and BETWITHIN (bw) were chose as methods to compute the denominator degrees of freedom (ddfm) (Schluchter and Elashoff, 1990; Kenward and Roger, 1997). Here we use serum IGF-I as an example to discuss the parameters chosen for SAS analysis. Sera from 5 HAY and 5 the CORN group cows were used to determine serum IGF-I concentration, so the numerator degrees of freedom (Num DF) for diet sequence (DietSeq) is 2 – 1 = 1, and ddfm is 2*(5 – 1) = 8 (approximately the number of cows). The Num DF for day or the interaction between diet sequence and day (DietSeq*Day) is 32, and ddfm is (330 – 1) – [(2 – 1) + (33 –1) + (2 – 1)*(33 – 1) + 2*(5 – 1)] = 256. As shown in the Appendix Table 1, the option kr has a too large ddfm for diet sequence, so BETWITHIN (bw) was chosen for SAS analysis.

2. Option for determining covariance structure

In SAS analysis, TYPE statement is used to define the covariance structure of days. For repeated measurements, Autoregressive(1) (ar(1)) or Heterogeneous AR(1) (arh(1)) is generally used as an option (Jennrich and Schluchter, 1986). The AR(1) specifies a first-order autoregressive structure, whereas ARH(1) specifies a heterogeneous first-order autoregressive structure (Wolfinger, 1996).

As described in the MATERIALS AND METHODS in chapter II, a blood sample was collected from each cow immediately before each GH administration and also every 24 hours following GH administration for a consecutive 7 d, and then once per week between two GH administrations, so the time interval between two consecutive samplings varied. Liver biopsy

was performed immediately before and the 7th day after each GH administration (i.e. d0, 7, 56, 63, 91 and 98), so the time interval between two consecutive samplings also varied. In SAS the Spatial Power (sp(pow)) structure is applicable for the unequally spaced data which can provide a direct generalisation of the AR(1) structure for equally spaced data. Therefore, Spatial Power (SP(POW)) is another option for these cow data analyses (Zimmerman and Harville, 1991).

As shown in the Appendix Table 1, the BIC Fit Statistic is roughly the same between ar(1), arh(1) and sp(pow). Together with the unequally spaced data, sp(pow) was selected for SAS analysis except body weight data.

The SAS code for PROC MIXED as following:

```
proc mixed data=original;
class DietSeq cow day;
model IGFI = DietSeq day DietSeq*day / ddfm=bw;
repeated day/subject= cow(DietSeq) type=sp(pow)(day) r rcorr;
* r: to request that blocks of the estimated R matrix be displayed;
* rcorr: to produce the correlation matrix corresponding to blocks of the estimated
R matrix, and strictly for selection of Type=;
lsmeans DietSeq*day / slice=day;
contrast ' Hay d0 vs d56' DietSeq 0 0 day 1 0 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 1 0 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0;
contrast ' Hay d56 vs d91' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
0 0 0 0 0 0 -1 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0
0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0;
contrast ' Hay d0 vs d91' DietSeq 0 0 day 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 -1 0 0 0 0 0 0 0 DietSeq*day 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0;
contrast ' Corn d0 vs d56' DietSeq 0 0 day 1 0 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0;
contrast ' Corn d56 vs d91' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0
0 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 -1
0 0 0 0 0 0 0;
contrast ' Corn d0 vs d91' DietSeq 0 0 day 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 -1 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 -1 0
0 0 0 0 0 0;
*these 6 contrasts were to determine the effect of feeding level;
contrast ' Hay d0 vs average d1-7' DietSeq 0 0 day 7 -1 -1 -1 -1 -1 -1 -1 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 7 -1 -1 -1 -1 -1 -1 -1 0
```

```
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0;
contrast ' Hay d56 vs average d57-63' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0
0 7 -1 -1 -1 -1 -1 -1 -1 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0
0 0 0 7 -1 -1 -1 -1 -1 -1 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0;
contrast ' Hay d91 vs average d92-98' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 7 -1 -1 -1 -1 -1 -1 -1 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 7 -1 -1 -1 -1 -1 -1 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0;
contrast ' Corn d0 vs average d1-7' DietSeq 0 0 day 7 -1 -1 -1 -1 -1 -1 -1 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 7 -1 -1 -1 -1 -1 -1 -1 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0;
contrast ' Corn d56 vs average d57-63' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0
0 7 -1 -1 -1 -1 -1 -1 -1 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 7 -1 -1
-1 -1 -1 -1 -1 0 0 0 0 0 0 0 0 0 0 0;
contrast ' Corn d91 vs average d92-98' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 7 -1 -1 -1 -1 -1 -1 -1 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 7 -1 -1 -1 -1 -1 -1 -1;
*these 6 contrasts were to determine the effect of GH administration;
contrast ' d7-d0: Hay vs corn ' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 1 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0;
contrast ' d63-d56: Hay vs corn ' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0
0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 -1 0 0 0 0 0 0 1
0 0 0 0 0 0 0 0 0 0 0;
contrast ' d92-d98: Hay vs corn' DietSeq 0 0 day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 DietSeq*day 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
0 -1 0 0 0 0 0 0 1;
* these 3 contrasts were to determine the effect of feeding level on GH response;
run;
```

3. Option chosen for cow body weight data

Body weight (BW) was recorded weekly on individual cows, so the time interval between two consecutive samplings was same. PROC CORR was used to determine the correlation structure of days. As shown in the Appendix Table 2 and 3, the correlation between days (that is, d0, d7, d14, d21, d28, d35, d42, d49, d56, d63, d70, d77, d84, d91 and d98) in the diet sequence HAY (0) or CORN (1) is similar to compound-symmetry structure, which has constant variance and constant correlation. Therefore, Compound Symmetry (cs) was chosen for SAS analysis.

The SAS code for PROC CORR as following:

```
proc corr data=original;
by DietSeq;
var d0 d7 d14 d21 d28 d35 d42 d49 d56 d63 d70 d77 d84 d91 d98;
run;
```

The SAS code for PROC MIXED as following:

```
proc mixed data=original;
class DietSeq cow day;
model BW = DietSeq day DietSeq*day / ddfm=sat;
repeated day/subject= cow(DietSeq) type=cs r rcorr;
lsmeans DietSeq*day / slice=day;
run;
```

Appendix Table 1. Selection of options for analysis of serum IGF-I concentration based on denominator degrees of freedom (ddfm) and covariance structure

	ddfm =	kr	kr	kr	bw	bw	bw
	type =	ar(1)	arh(1)	sp(pow)	ar(1)	arh(1)	sp(pow)
Effect	Num DF	Denominator DF for F-test					
DietSeq	1	24.2	18.6	73.6	8	8	8
Day	32	237	54.3	240	256	256	256
DietSeq*day	32	237	54.3	240	256	256	256
BIC Fit Statistic		2895	2846	2908	2895	2846	2908

Note: Kenward-Rogers (kr); BETWITHIN (bw); Numerator degrees of freedom (Num DF); Autoregressive(1) (ar(1)); Heterogeneous AR(1) (arh(1)); Spatial Power (sp(pow)).

Appendix Table 2. Pearson Correlation Coefficients between days for body weight in the HAY group of cows [1]

	d0	d7	d14	d21	d28	d35	d42	d49	d56	d63	d70	d77	d84	d91	d98
d0	1	0.94	0.89	0.93	0.94	0.94	0.94	0.91	0.87	0.94	0.89	0.95	0.87	0.94	0.86
		<.0001	0.0005	<.0001	<.0001	<.0001	<.0001	0.0003	0.001	<.0001	0.0004	<.0001	0.0008	<.0001	0.0011
d7		1	0.96	0.97	0.95	0.97	0.94	0.97	0.86	0.96	0.94	0.94	0.91	0.94	0.89
			<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0012	<.0001	<.0001	<.0001	0.0002	<.0001	0.0004
d14			1	0.98	0.95	0.95	0.91	0.93	0.87	0.94	0.94	0.9	0.89	0.92	0.91
				<.0001	<.0001	<.0001	0.0002	<.0001	0.0009	<.0001	<.0001	0.0003	0.0004	0.0001	0.0002
d21				1	0.98	0.98	0.96	0.96	0.93	0.97	0.96	0.95	0.93	0.96	0.94
					<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
d28					1	0.97	0.97	0.95	0.9	0.96	0.92	0.95	0.89	0.97	0.91
						<.0001	<.0001	<.0001	0.0004	<.0001	0.0001	<.0001	0.0004	<.0001	0.0002
d35						1	0.97	0.98	0.9	0.97	0.97	0.98	0.95	0.98	0.92
							<.0001	<.0001	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001
d42							1	0.97	0.94	0.98	0.93	0.98	0.93	0.97	0.94
								<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
d49								1	0.9	0.96	0.95	0.95	0.93	0.95	0.9
									0.0004	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003
d56									1	0.94	0.9	0.93	0.91	0.91	0.93
										<.0001	0.0003	<.0001	0.0002	0.0002	<.0001
d63										1	0.92	0.95	0.92	0.94	0.93
											0.0001	<.0001	0.0002	<.0001	<.0001
d70											1	0.96	0.97	0.95	0.91
												<.0001	<.0001	<.0001	0.0002
d77												1	0.96	0.98	0.93
													<.0001	<.0001	<.0001
d84													1	0.95	0.94
														<.0001	<.0001
d91														1	0.95
															<.0001
d98															1

[1] Note: constant correlations across each row.

Appendix Table 3. Pearson Correlation Coefficients between days for body weight in the CORN group of cows [1]

	d0	d7	d14	d21	d28	d35	d42	d49	d56	d63	d70	d77	d84	d91	d98
d0	1	0.89	0.77	0.82	0.77	0.78	0.75	0.69	0.68	0.71	0.7	0.65	0.71	0.66	0.6
		0.0005	0.0079	0.0032	0.0081	0.0071	0.0124	0.0262	0.0280	0.0202	0.0224	0.0414	0.0193	0.0377	0.0645
d7		1	0.79	0.9	0.85	0.86	0.8	0.79	0.78	0.85	0.82	0.8	0.86	0.82	0.75
			0.0062	0.0003	0.0015	0.0012	0.0046	0.0056	0.0076	0.0018	0.0032	0.0054	0.0012	0.0034	0.0123
d14			1	0.93	0.94	0.85	0.95	0.92	0.95	0.92	0.92	0.9	0.9	0.84	0.89
				<.0001	<.0001	0.0014	<.0001	0.0001	<.0001	0.0001	0.0001	0.0003	0.0004	0.0019	0.0005
d21				1	0.96	0.94	0.97	0.96	0.95	0.96	0.96	0.93	0.94	0.89	0.89
					<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0004	0.0004
d28					1	0.92	0.96	0.95	0.97	0.96	0.98	0.96	0.96	0.88	0.9
						0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0006	0.0004
d35						1	0.9	0.89	0.89	0.94	0.95	0.93	0.94	0.92	0.89
							0.0004	0.0004	0.0006	<.0001	<.0001	<.0001	<.0001	0.0002	0.0005
d42							1	0.98	0.98	0.94	0.95	0.92	0.92	0.86	0.9
								<.0001	<.0001	<.0001	<.0001	0.0001	0.0001	0.0012	0.0003
d49								1	0.97	0.96	0.95	0.94	0.94	0.89	0.93
									<.0001	<.0001	<.0001	<.0001	<.0001	0.0005	<.0001
d56									1	0.95	0.96	0.95	0.94	0.89	0.91
										<.0001	<.0001	<.0001	<.0001	0.0005	0.0002
d63										1	0.98	0.98	0.98	0.96	0.95
											<.0001	<.0001	<.0001	<.0001	<.0001
d70											1	0.99	0.98	0.92	0.94
												<.0001	<.0001	0.0001	<.0001
d77												1	0.97	0.93	0.95
													<.0001	<.0001	<.0001
d84													1	0.96	0.96
														<.0001	<.0001
d91														1	0.94
															<.0001
d98															1

[1] Note: constant correlations across each row.

Appendix Table 4. Dependent variables were measured on various days.

Dependent variables included body weight, serum IGF-I concentration, serum GH concentration, IGF-I stability in serum, serum IGFBP-3 abundance, serum ALS abundance, liver IGF-I mRNA abundance, liver IGFBP-3 mRNA abundance, liver ALS mRNA abundance, liver ALS protein abundance (l-ALS), liver IGF-I polysomal mRNA abundance (ps IGF-I), liver IGFBP-3polysomal mRNA abundance (ps IGFBP-3), and liver ALS polysomal mRNA abundance (ps ALS).

	day																														
Variable	0	1	2	3	4	5	6	7	14	21	28	35	42	49	56	57	58	59	60	61	62	63	70	77	84	91	92	93	94	95	96	97	98
Body weight	*							*	*	*	*	*	*	*	*							*	*	*	*	*							*
Serum IGF-I	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Serum GH	*	*	*	*	*	*	*	*	*	*					*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*
IGF-I stability	*	*						*	*					*	*	*		*				*	*			*	*						*
Serum IGFBP-3	*	*		*				*	*					*	*	*		*				*	*			*	*						*
Serum ALS	*	*		*				*	*					*	*	*		*				*	*			*	*						*
IGF-I mRNA	*							*							*							*				*							*
IGFBP-3 mRNA	*							*							*							*				*							*
ALS mRNA	*							*							*							*				*							*
l-ALS	*							*							*							*				*							*
ps IGF-I	*							*							*							*											
ps IGFBP-3	*							*							*							*											
ps ALS	*							*							*							*											

Appendix Table 5. Expected ANOVA

Source	DietSeq		day		DietSeq*day	
Variable	Num DF	Denominator DF	Num DF	Denominator DF	Num DF	Denominator DF
Change in body weight	1	18	14	252	14	252
Serum IGF-I	1	8	32	256	32	256
Serum GH	1	8	27	216	27	216
IGF-I stability in serum	1	8	12	96	12	96
Serum IGFBP-3	1	7	13	91	13	91
Serum ALS	1	8	13	104	13	104
Liver IGF-I mRNA	1	8	5	35	5	35
Liver IGFBP-3 mRNA	1	8	5	40	5	40
Liver ALS mRNA	1	8	5	40	5	40
Liver ALS protein	1	8	5	40	5	40
liver IGF-I polysomal mRNA	1	8	3	16	1	16
liver IGFBP-3 polysomal mRNA	1	8	3	16	1	16
liver ALS polysomal mRNA	1	8	3	16	1	16

APPENDIX II

Effects of feeding level and GH administration on liver IGF-I mRNA expression

Another RPA assay was conducted to verify liver IGF-I mRNA abundance as shown in Figure 2-6. Liver total RNAs of 7 HAY and 7 the CORN group cows were hybridized with α-^{32}P-labeled probes for bovine IGF-I and bovine GAPDH mRNAs, digested by RNase A/T1, and then resolved by electrophoresis on 6% acrylamide gels.

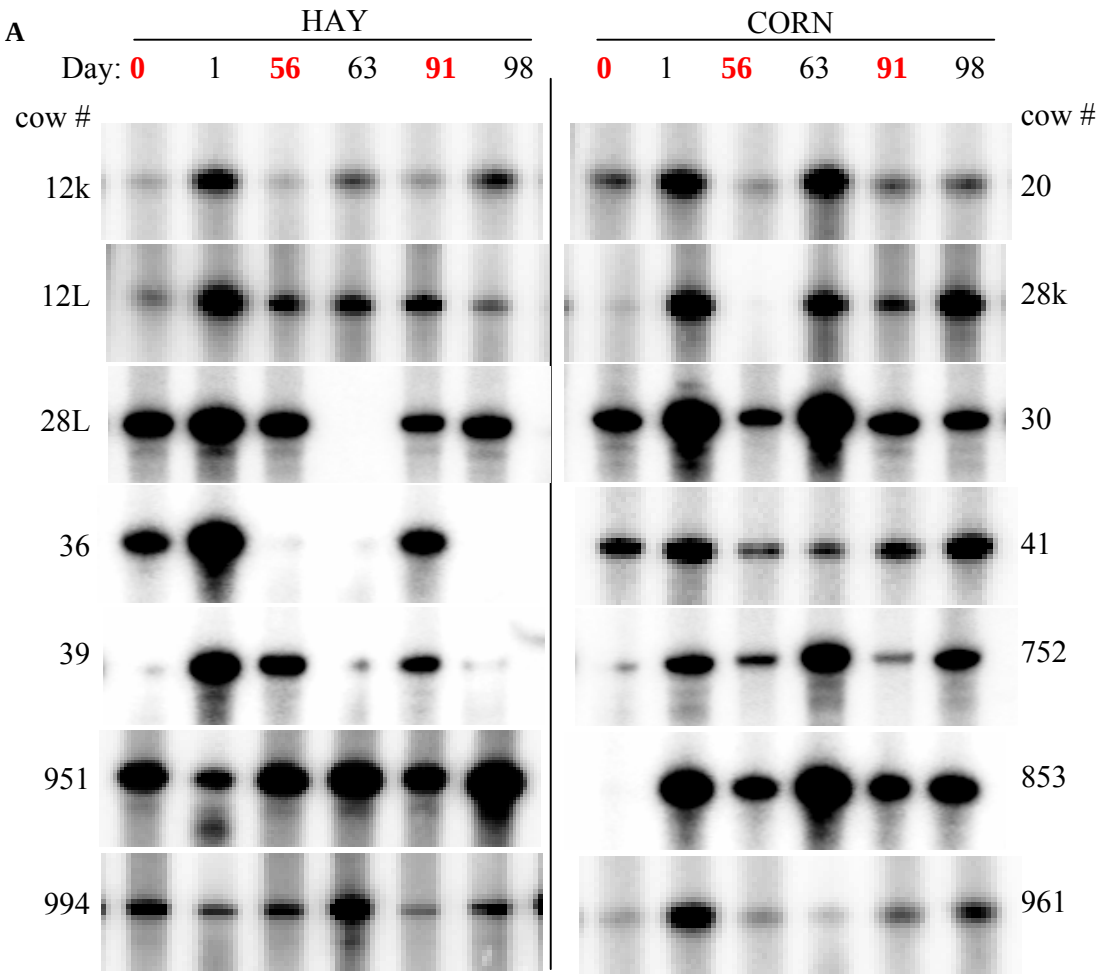
Liver IGF-I mRNA abundance was not different between diet sequences ($P = 0.40$), and there was also not an interaction of diet sequence with day ($P = 0.24$). However, there was an effect of day ($P < 0.01$). As shown in Figure 4-1A and 4-4C, basal liver IGF-I mRNA abundance was not different between d0, d56 and d91 in the HAY group cows ($P = 0.99$, 0.99 and 0.99, respectively) or in the CORN group cows ($P = 0.99$, 0.99 and 0.99, respectively), neither was it between the HAY and CORN group cows on d0, d56 or d91 ($P = 0.66$, 0.24 and 0.50 respectively). These results are consistent with those in Figure 2-6, indicating that supplementation of concentrates or moderate food restriction does not alter liver IGF-I mRNA abundance prior to GH administration.

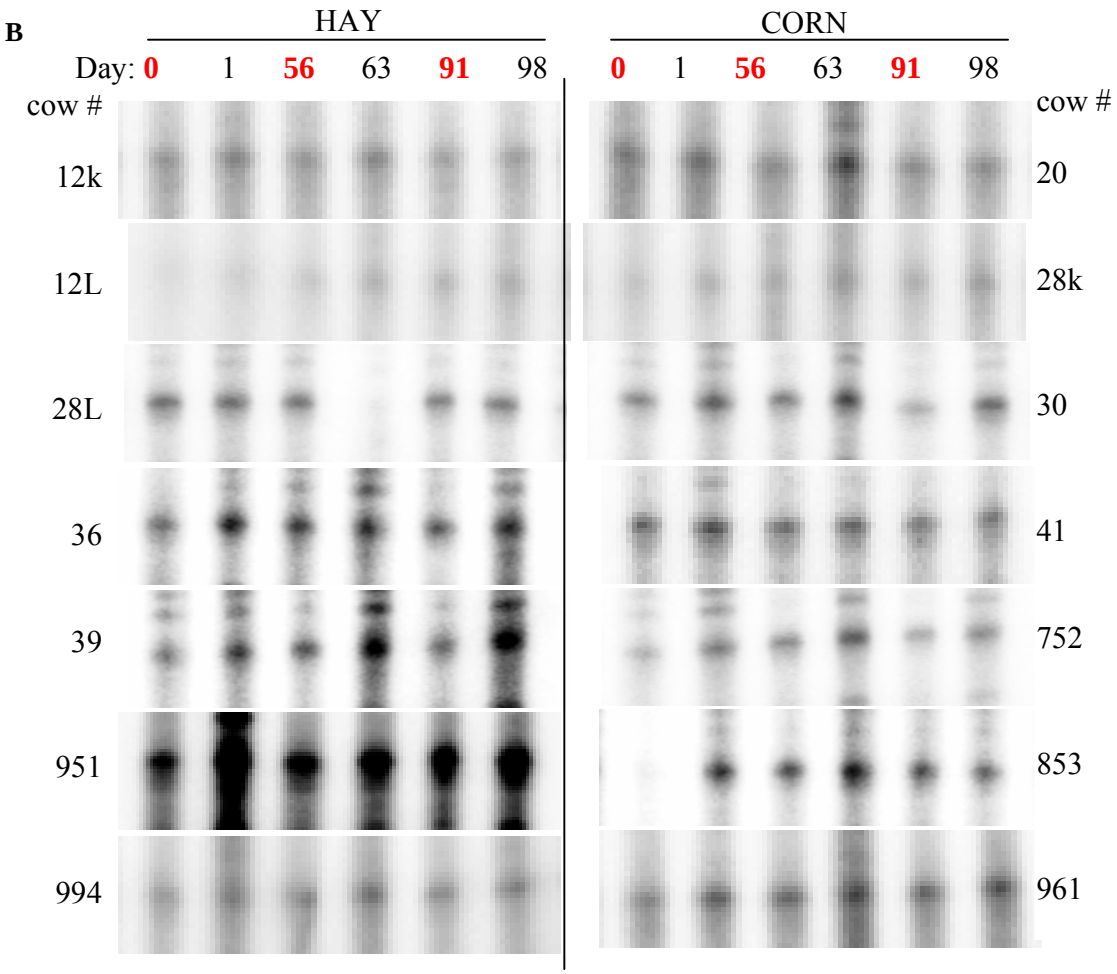
Seven days of GH administration increased liver IGF-I mRNA abundance in the HAY group cows on d7 ($P < 0.05$), but not on d63 or d98 ($P = 0.95$ and 0.76 respectively). It also increased liver IGF-I mRNA abundance in the CORN group cows on d7 and d63 ($P < 0.01$ and < 0.01 respectively), but not on d98 ($P = 0.56$). There was no difference in GH-stimulated liver IGF-I mRNA abundance between the HAY and CORN group cows on d7, d63 and d98 ($P = 0.49$, 0.065 and 0.50, respectively), or in the GH-stimulated increase in liver IGF-I mRNA abundance between the HAY and CORN group cows from d0 to d7, from d56 to d63, or from d91 to d98 (P

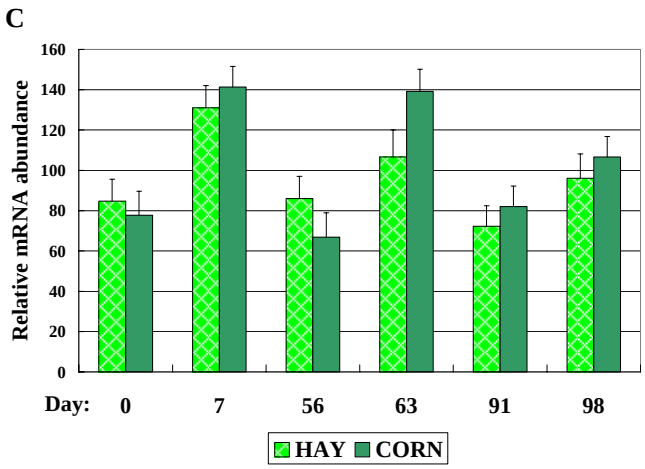
= 0.99, 0.24 and 0.99, respectively). These results are consistent with those in Figure 2-6, indicating that GH administration increased liver IGF-I mRNA expression, but the increase was not affected by feeding level.

Figure 4-1. Liver IGF-I mRNA levels in cows.

Liver total RNA of 7 HAY and 7 CORN group cows was hybridized with α-^{32}P-labeled probes for bovine IGF-I and GAPDH mRNAs, digested by RNase A/T1, and then resolved by electrophoresis on 6% acrylamide gels. **A:** RPA images of liver total IGF-I mRNA in 7 CORN and 7 the HAY group cows. **B:** RPA images of loading control liver GAPDH mRNA in 7 CORN and 7 the HAY group cows. **C:** Densitometric quantification of ribonuclease-protected mRNA bands in panel A. Each bar represents LS mean ± SE. Effects of DietSeq and the interaction between diet sequence and day were not different ($P = 0.40$ and 0.24, respectively), and there was an effect of day ($P < 0.01$). Following 7 days of GH administration, liver IGF-I mRNA abundance was increased in the HAY group cows on d7 ($P < 0.05$), and in the CORN group cows on both d7 and d63 ($P < 0.01$ and < 0.01, respectively).


A
HAY
CORN
Day: 0 1 56 63 91 98 0 1 56 63 91 98
cow #
12k
12L
28L
36
39
951
994
cow #
20
28k
30
41
752
853
961


B
HAY
CORN
Day: 0 1 56 63 91 98
0 1 56 63 91 98
cow #
12k
12L
28L
36
39
951
994
cow #
20
28k
30
41
752
853
961


C
Relative mRNA abundance
160
140
120
100
80
60
40
20
0
Day: 0 7 56 63 91 98
HAY CORN

Vita

Miaozong Wu, son of Guoqing Wu and Huojin Zhan, is originally from Anxi, Fujian, P. R. China. He graduated from China Agricultural University with a Bachelor of Science degree in Animal Biochemistry and Physiology in 1996 and Chinese Academy of Agricultural Sciences with a Master of Science degree in Animal Nutrition and Feed Science in 1999. He worked at the Feed Research Institute, Chinese Academy of Agricultural Sciences from 1999 to 2004. He started his Ph. D. program under the direction of Dr. H. Jiang at Virginia Polytechnic Institute and State University in August, 2004 and defended on Augest 31, 2007. He was awarded the John Lee Pratt Animal Nutrition Fellowship from 2004 to 2007.