

The Relationships between Plasma Insulin-like Growth Factor (IGF)-1 and IGF-Binding Proteins (IGFBPs) to Growth Pattern, and Characteristics of Plasma IGFBPs in Steers*

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ABSTRACT : This study was conducted to determine the characteristics of IGFBPs in plasma of steers, and to profile the relationship between growth and plasma IGF-1 and IGFBPs with aging in Holstein steers. Four blots of IGFBP at molecular weights of 38-43, 34, 29-32 and 24 kDa bands were detected by western ligand blot assay using ¹²⁵I-IGF-1. On the basis of immunoblotting with anti-bovine IGFBP-2 and -3 antisera, we observed the band for IGFBP-2 at approximately 34 kDa, and the IGFBP-3 band was detected at 38-43 kDa and 34 kDa in adult steers and calves. The IGFBP-3 antiserum used on the blots exhibited significant cross-reactivity with 34 kDa IGFBP-2. Furthermore, the 38-43 kDa IGFBP-3 bands were reduced to a 36 kDa band after deglycosylation, whereas the 34 kDa IGFBP-2 was intact. The plasma IGF-1, IGFBP-3 and other IGFBPs showed stability throughout a whole day. The change in live weight was found to be positively correlated to the plasma IGF-1 concentration ($r = 0.6801$, $n = 64$, $p < 0.05$) and plasma IGFBP-3 ($r = 0.6321$, $n = 64$, $p < 0.05$), while inversely correlated to plasma IGFBP-2 ($r = -0.2919$, $n = 64$, $p < 0.05$). Furthermore, plasma IGF-1 was positively correlated to plasma IGFBP-3 ($r = 0.6191$, $p < 0.001$), but was not correlated to plasma IGFBP-2. The portion of IGFBP-2 for total IGFBPs in calves was higher than in adult steers ($p < 0.05$) and was decreased with growth, whereas that of IGFBP-3 was increased with increased live weight ($p < 0.05$). The ratio IGFBP-3 for IGFBP-2 (BP-3/BP-2) was increased with growing of liveweight. Therefore, the changes in plasma IGF-1 level with increased liveweight may be related to the changes in plasma IGFBP-3 level and IGFBP-2 may give an important role in anabolic action of IGF-1 with the growth of body during calthood in Holstein steers. (*Asian-Aust. J. Anim. Sci.* 2005, Vol 18, No. 11 : 1575-1581)

Key Words : IGF-1, IGFBPs, Growth Stages, Steer

INTRODUCTION

Insulin-like growth factor (IGF)-1 is a growth promoting peptide that is related to growth and body size in animals (Hossner et al., 1997). It has been noted as a predictor of growth and nutritional status for the domestic animal industry. In ruminants, circulating IGF-1 is positively correlated with growth (Ronge and Blum 1989; Roberts et al., 1990) and growth rate (Bishop et al., 1991). This relationship may be due to the mitogenic effect of IGF-1 (Bark et al., 1998; Davis et al., 2002). Mitogenic activity is modulated by IGF-binding proteins (IGFBPs) because IGF-1 in the blood is almost completely complexed with high affinity IGFBPs (Zapf, 1995). IGFBPs have a role in the maintenance of stable concentrations of IGF-1 (Guler et al., 1989), regulation of IGF-1 clearance (Blum and Ranke, 1990) transport of IGF-1 from serum to target tissue (Bar et al., 1990) and modulation of IGF-1 action at the target

receptor at the cell membrane (Argente et al., 1993; Zapf, 1995).

In humans, serum IGFBP-3 increases until puberty and then continuously decreases with age in adults, the pattern shows a positive correlation with IGF-1 (Argente et al., 1993; Juul et al., 1995; Gomez et al., 2003). In contrast to IGFBP-3, serum IGFBP-2 shows high levels at birth and low levels during puberty and a negative correlation with IGF-1 (Schwander and Mary, 1993; Rajaram et al., 1997). The changes in serum concentrations of the various IGFBPs with increased IGF-1 during puberty may be related to the stimulated growth at this stage. This provides evidence that blood IGFBPs are related to the change in IGF-1 with an increase in liveweight in animals.

Therefore, understanding the characteristics of IGFBPs, and the relationship between IGF-1 and IGFBPs in the blood to growth will provide the basic information necessary to clarify the role plasma IGFBPs play in regulating IGF-1 during growth in steers.

The objectives of this study are 1) to determine the characteristics of IGFBPs in steer plasma, 2) to understand the relationship between growth and plasma IGF-1 and IGFBPs in steers.

MATERIALS AND METHODS

Animals and experimental design deglycosylation and detection of IGFBPs

* This study was supported by the Brain Korea 21 program and a grant from BioGreen 21 program (20050301-034-445-009-02-00), Rural Development Administration, Republic of Korea.

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Received December 16, 2004; Accepted May 25, 2005

The plasma of four 18 month-old Holstein adult steers and four 4 month-old Holstein steer calves were used in this experiment. The experimental animals ($n = 8$) were castrated at the age of 3 months (approximately 120 kg). Average body weights of adult steers ($n = 4$) and calves ($n = 4$) were $597 \text{ kg} \pm 13 \text{ SEM}$ and $137 \text{ kg} \pm 11 \text{ SEM}$, respectively. The adult steers were offered hay and concentrate diet with 1.6% DM (CP 12.5% TDN 75%) per day of body weight twice daily at 900 and 1700 h. The calves were offered hay and concentrate diet with 2.6% DM (CP 17.2% TDN 77%) of body weight twice daily at 900 and 1700 h. The diets were formulated based on recommendation of the NRC (NRC, 1998). Blood samples were taken through the jugular vein catheter for 1 h with 20-min intervals from 0800 to 0900, from 1200 to 1300 and from 1600 to 1700. Twelve samples collected for one day were pooled for deglycosylation, immunoblotting and ligand blotting bands of IGFBPs. The collected blood was centrifuged and plasma samples were stored at -30°C until assayed.

Twenty four-h profile of plasma IGF-1 and IGFBPs

Eight 13 month old Holstein steers were used for the experiment. Average body weight was $294 \text{ kg} \pm 5 \text{ SEM}$. The experimental animals were housed in a pen and fed a hay and concentrate diet with 2.0% of body weight dry matter (DM) based from 0830 h to 0930 h and from 1700 h to 1800 h twice daily. Water was available continuously. The CP and TDN concentration of the rations were 11.85% and 71.15%. Blood samples were collected through the jugular vein catheter at 1 h intervals for 24 h for analysis of IGF-1 and IGFBPs.

Changes in plasma IGF-1 and IGFBPs with growth

Eight Holstein steers were used for the experiment. The experiment was conducted for 26 months. The experimental animals were housed in pen and fed a hay and concentrate diet twice daily. The experimental animals were castrated at the age of 3 months (approximately 120 kg). The ration was formulated and given by recommendation of the NRC (NRC, 1998). The blood samples were taken at 50-100, 100-150, 200-250, 300-350, 400-450, 500-550, 600-650 and $650 \text{ kg} < \text{body weight}$ for IGF-1 and IGFBPs analysis.

All animal-based procedures were in accordance with the "Guidelines for the Care and Use of Experimental Animals of Seoul National University", which were formulated from the "Declaration of Helsinki and Guiding Principles in the Care and Use of Animals".

Analysis of plasma IGF-1

Plasma concentrations of IGF-1 were determined by radioimmunoassay (RIA) as described previously (Lee et al., 2000). For the IGF-1 assay, recombinant human IGF-1 (lot #30) purchased from Amersham bioscience (Uppsala,

Sweden) was used for standard. The labeled ^{125}I -hIGF-1 (code IM172) was purchased from Amersham bioscience (Uppsala, Sweden). The specific activity averaged $30 \mu\text{Ci}$. AFP4892898 anti-human-IGF-1 (first antibody) was a gift from Dr A. F. Parlow (National Hormone and Peptide Program, Torrance, CA). Goat anti-rabbit antiserum (second antibody) was purchased from Sigma Chemical Company (St Louis, MO, USA).

The plasma samples were first extracted according to the method of Daughaday et al. (1980). The sensitivity of the IGF-1 assay was 0.73 ng/ml , and inter- and intra-assay CV were 14.6 and 8.2%, respectively.

Analysis of plasma IGFBPs

Western-Ligand blotting : Analysis of IGFBPs was performed using a Western ligand blot procedure (Lee et al., 2000). Twelve point five % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed under nonreducing conditions according to the procedure of Laemmli (Laemmli, 1970). After electrophoresis. Proteins were electro transferred to a nitrocellulose membrane at 30 V at room temperature for 3 h. Detection of the IGFBPs was performed according to Hossenlopp et al. (1986). The intensities on the autoradiographs were analyzed using NIH-image V1.61 software (NIH: National Institutes of Health, USA). The data of IGFBPs bands were expressed as a percentage of the relative abundance of each sample IGFBP band compared to the each IGFBP bands of standard plasma pool as determined by scanning (NIH-image) of autoradiogram from Western-ligand blot.

Immunoblotting : Immunoassay for detection of the IGFBPs was performed according to the procedure of Funston et al. (1995). Membranes were incubated in western immunoblot buffer (0.1 M Tris base, 0.15 M NaCl, and 0.1% Triton-X 100 pH 7.5) for 30 min at room temperature and probed for 2 h with the following polyclonal antisera: rabbit anti-bovine IGFBP-2 (lot 12415; Upstate Biotechnology, Lake Placid, NY) or rabbit anti-bovine IGFBP-3 (kindly provided by Dr. D. R. Clemmons, University of North Carolina, Chapel Hill, NC) diluted at 1:1,000 in immunoblot buffer. The blots were incubated for 1 h at room temperature in immunoblot buffer containing peroxidase-conjugated goat antirabbit immunoglobulin G secondary antibody (Sigma Chemical, St. Louis, MO). Excess secondary antibody was removed by rinsing twice in immunoblot buffer, and substrate solution was added and then incubated for 10 sec at room temperature. The substrate solution was a mixture of 5 ml 4-chloro-1-naphthol stock solution (3 mg/ml MeOH), 20 ml 50 mM Tris-HCl (pH 7.5) and 25 μl 5% H_2O_2 . The filter membrane was washed once with distilled water and dried between filter paper and stored. The antiserum to bovine IGFBP-3 used in

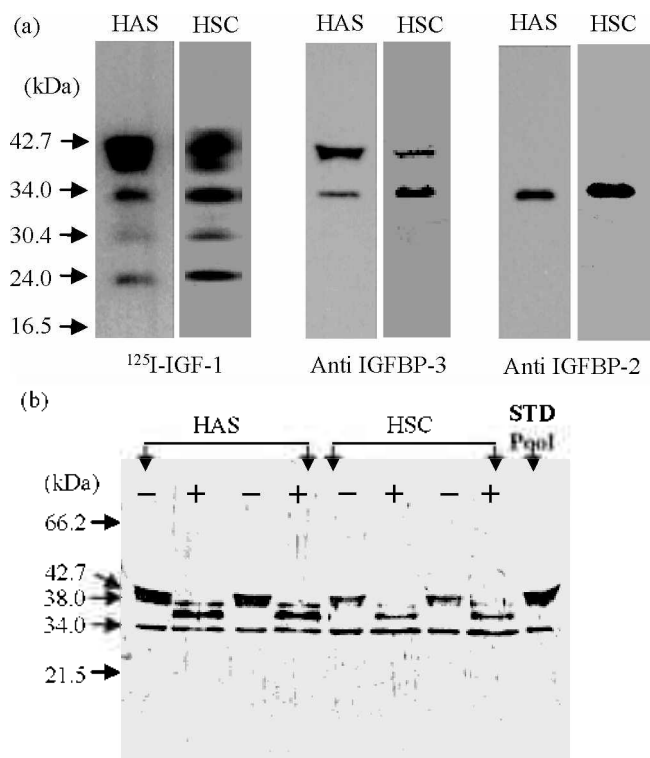


Figure 1. (a) Western ligand and immunoblots in Holstein adult steers. One μl plasma was run on a 12.5% SDS-PAGE gel, transferred to nitrocellulose membrane and incubated with 100,000 cpm ^{125}I -IGF-1/10 ml buffer for ligand blots and with bovine antisera to IGFBP-2 and IGFBP-3 for immunoblots, and the IGFBP-3 antiserum used on blot exhibits significant cross reactivity with IGFBP-2 (b) deglycosylation of IGFBP-2 and IGFBP-3 in Holstein adult steer (HAS) and calves (HSC). Blots were coincubated with bovine antisera to IGFBP-2 and IGFBP-3. The molecular weight markers used were BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

these experiments cross-reacts with IGFBP-2 (Colick and Clemmons, 1991).

Deglycosylation of IGFBP-2 and IGFBP-3 : Deglycosylation of plasma proteins was performed using N-glycanase (Glycopeptidase-F, lot LEK7431, Wako Chemical, Japan) according to the procedure of (Funston et al., 1996). One μl plasma was incubated with 3 U N-glycanase at 37°C for 24 h in 30 μl (total volume) 0.3 M sodium phosphate buffer, pH 8.6, containing 1.25% Triton X-100, 0.17% SDS, and 5.75 μM phenylmethylsulfonyl-fluoride. After incubation, the reaction mixture was mixed with an equal volume of SDS-gel sample buffer, boiled for 5 min, and subjected to the Western immunoblotting procedure.

Statistical analysis

Mean values and the standard error of the means were calculated. The twenty four-h profile data of IGF-1 and

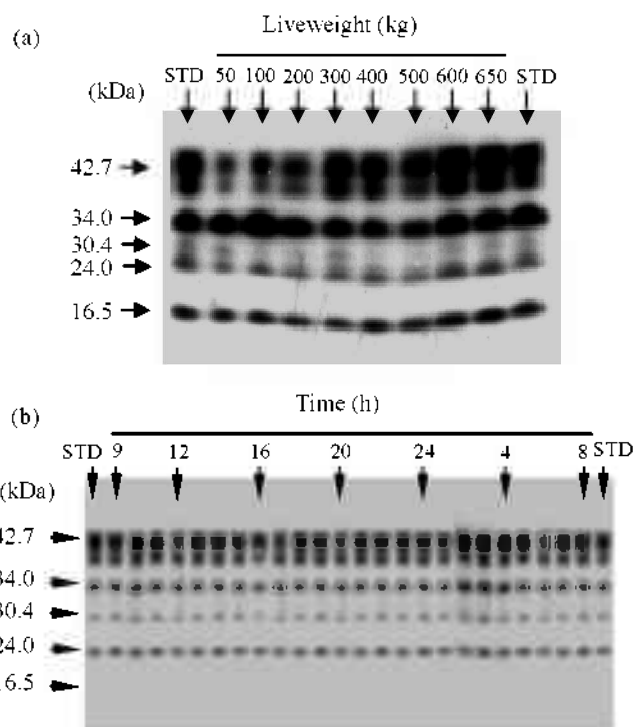


Figure 2. (a) The change in Western ligand blots of plasma IGFBPs with growing in Holstein steers. The 64 plasma samples collected during eight-stage from eight steers were pooled as standard plasma (STD)(lane1). (b) The change in Western ligand blots of plasma IGFBPs during twenty four-h in Holstein steers. The 192 plasma samples collected for twenty four-hr from eight steers were pooled as standard plasma (STD)(lane1 or 26). Apparent molecular weights (MW) of IGFBPs are expressed from comparison with pre-stained standards run on same gel. The molecular weight markers used were BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

IGFBPs were analyzed by analysis of variance, and the Duncan's multiple range test using General Linear Model (GLM) procedure of the SAS program package (SAS Institute, Cary, NC). The profile data of liveweight, IGF-1 and IGFBPs with growing were analyzed the Dunnett's T test using GLM procedure for variable. The correlation and partial correlation coefficients were calculated by linear regression (REG) and assessed for significance using Fisher's test.

RESULTS

Glycosylation and detection of IGFBPs

In ligand blotting assay of the plasma, the four bands of IGFBP obtained had molecular weights of 38-43, 34, 29-32 and 24 kDa (Figure 1a). On the basis of immunoblotting with anti-bovine IGFBP-2 and -3 antisera, the band for IGFBP-2 was observed at approximately 34 kDa, and the IGFBP-3 bands were detected at 38-43 kDa and 34 kDa

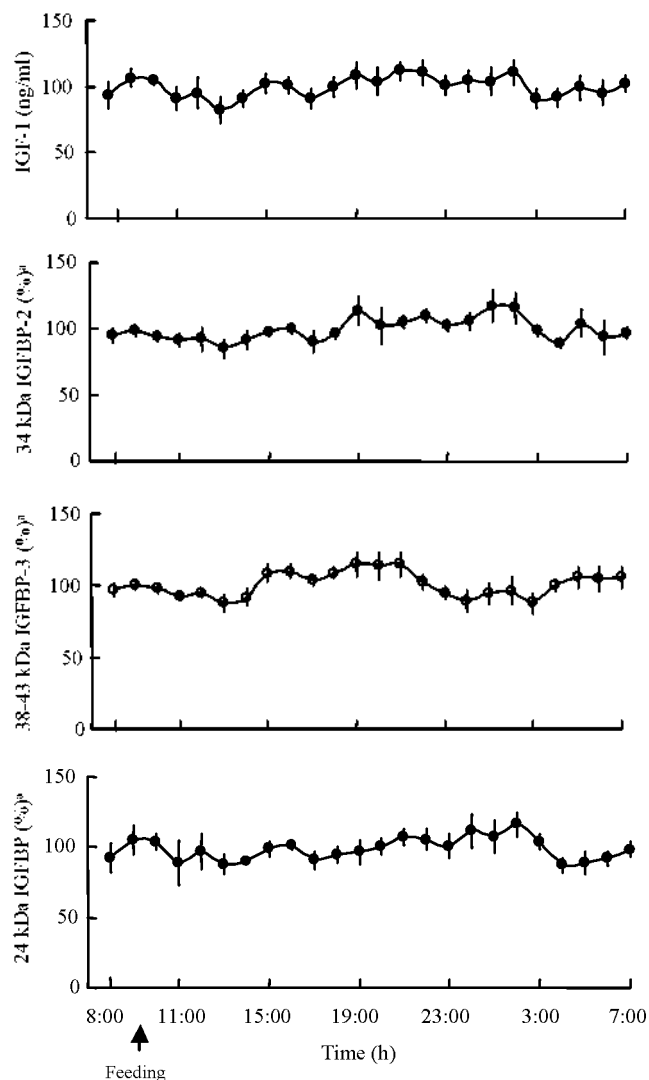


Figure 3. Twenty four-hr profile of plasma IGF-1 and IGFBPs levels in Holstein steers. Data points represent mean \pm SEM for eight steers. ^{a)} was expressed as a relative percentage of the absorbance of that IGFBP band for abundance of pooled standard plasma as determined by scanning (NIH-image V1.61 software) of autoradiogram from western-ligand blot.

in adult steers and calves (Figure 1a). The IGFBP-3 antiserum used for the blots exhibited significant cross-reactivity with 34 kDa IGFBP-2 (Figure 1a, b). The 24 kDa bands were putatively determined as IGFBP-4 by comparing them with bands in previous reports that used IGFBP-4 for the 24 kDa band in an immunological assay (Funston et al., 1996). When the plasma was treated with N-glycanase before electrophoresis and immunoblotting, the 38-43 kDa IGFBP-3 bands moved to 36.5-38 kDa bands after deglycosylation, whereas the 34 kDa IGFBP band was unaffected in adult steers and calves (Figure 1b).

Twenty four-h profile of plasma IGF-1 and IGFBPs

Plasma IGF-1 and IGFBPs concentrations monitored with one-h interval for twenty four-h in growing Holstein

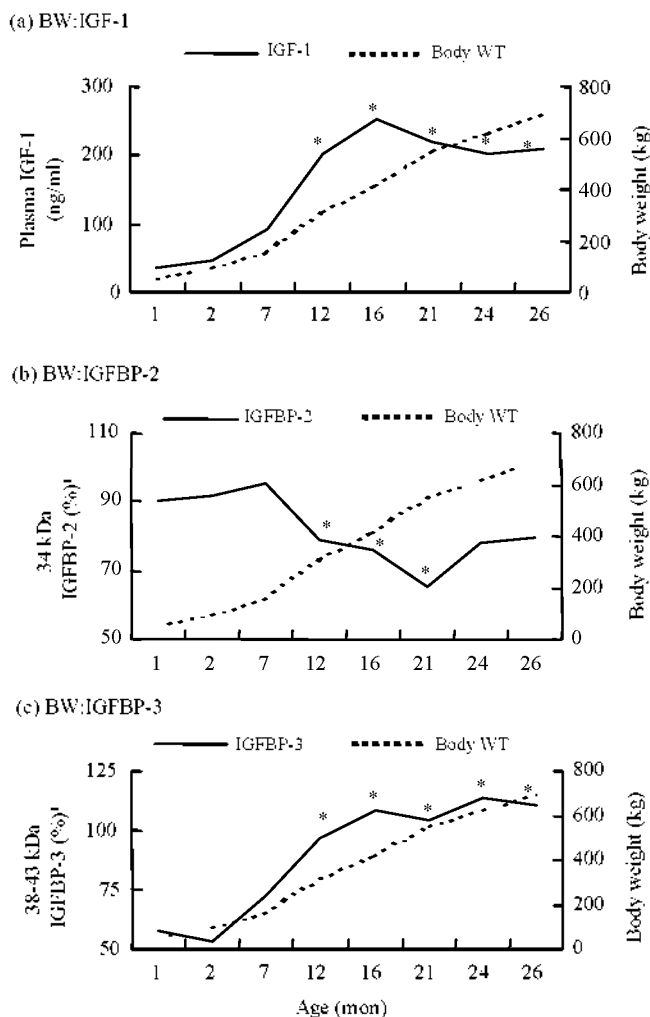


Figure 4. The change in plasma IGF-1 and IGFBPs with growing in Holstein steers. Data points represent mean for eight steers. ¹ was expressed as a relative percentage of the abundance of that IGFBP band for abundance of pooled standard plasma as determined by scanning (NIH-image V1.61 software) of autoradiogram from western-ligand blot. * $p < 0.05$, compared with 1 mon (50-100 kg BW).

steers, were not significantly different. On the basis of ligand blotting, when the data of IGFBPs bands were expressed as a percentage of the relative abundance of each sample IGFBP band compared to the each IGFBPs bands of standard plasma pool (Figure 2b and 3).

Relationship of plasma IGF-1 and IGFBPs with growth

Plasma IGF-1 concentration (Figure 4a) was gradually increased with age until 12 m (300-350 kg) ($p < 0.05$), but it was not change after 400 kg. Plasma IGFBP-2 (Figure 2a, 4b) was decreased from 12 m (300-350 kg) to 21 m (500-550 kg) ($p < 0.05$). Plasma IGFBP-3 (Figure 2a, 4c) was increased from 7 m and the concentrations at 12 m were significantly different compared to those at 1 m (50-100 kg) ($p < 0.05$). However, the concentrations of other IGFBPs were not changed with growth. The ratio of IGFBP-3 to

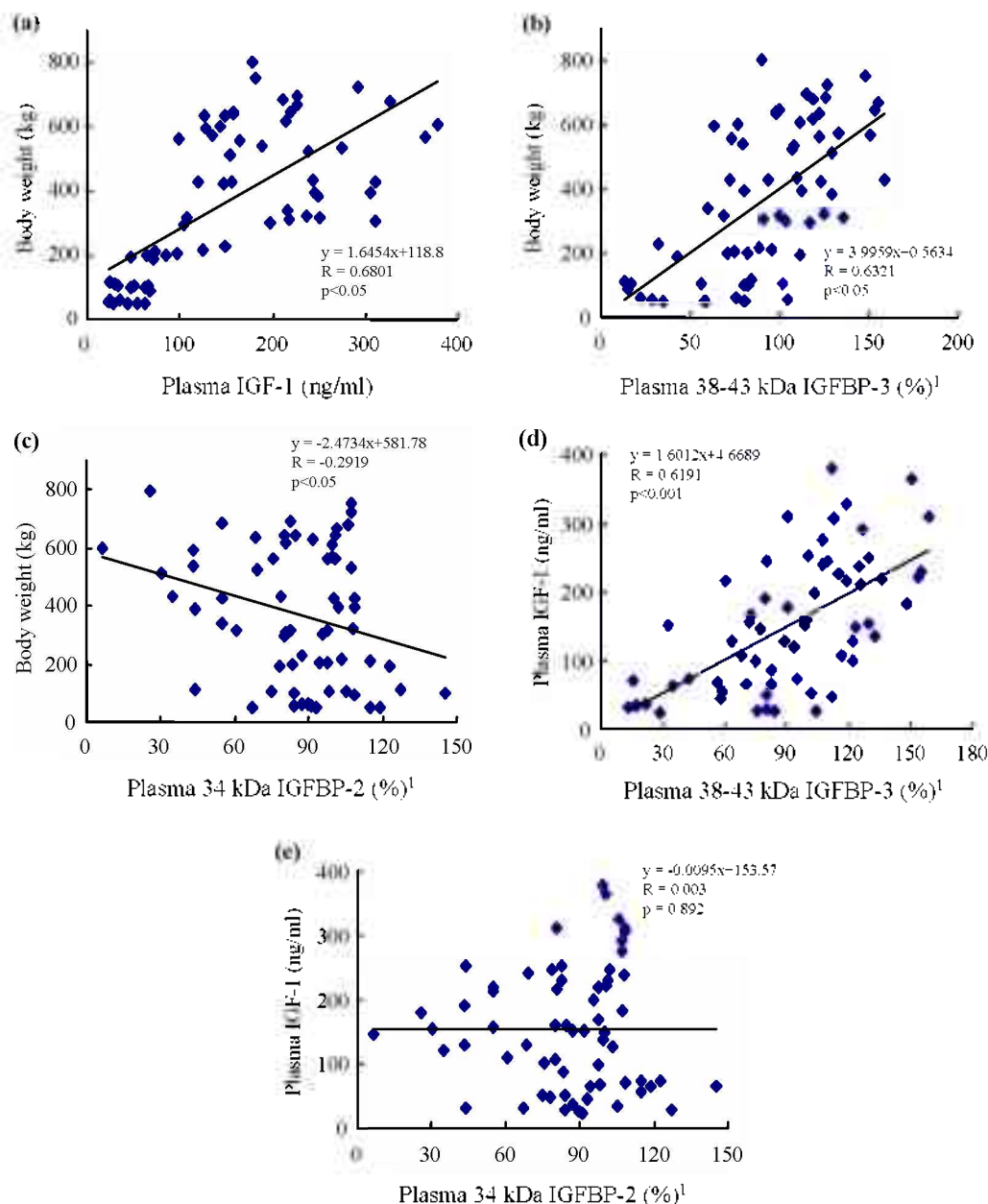


Figure 5. Relationship between liveweight and plasma IGF-1 and IGFBPs levels with growing in Holstein steers. Data represent mean for eight steers. ¹ was expressed as a relative percentage of the abundance of that IGFBP band for abundance of pooled standard plasma as determined by scanning (NIH-image V1.61 software) of autoradiogram from western-ligand blot.

IGFBP-2 (IGFBP-3/IGFBP-2) was increased with the increase in liveweight from 16 m (300-35 kg). The changes in body weight were found to be positively correlated with the plasma IGF-1 ($r = 0.6801$, $n = 64$, $p < 0.05$) (Figure 5a) and plasma IGFBP-3 concentrations ($r = 0.6321$, $n = 64$, $p < 0.05$) (Figure 5b), while these were inversely correlated with IGFBP-2 ($r = -0.2919$, $n = 64$, $p < 0.05$) (Figure 4c). Furthermore, plasma IGF-1 was positively correlated with plasma IGFBP-3 ($r = 0.6191$, $n = 64$, $p < 0.001$) (Figure 5d), but not correlated to IGFBP-2 (Figure 5e). However, no relationships were observed between other IGFBPs and plasma IGF-1 with growth.

DISCUSSION

The present study detected four blots of IGFBP at molecular weights of 38-43, 34, 29-32 and 24 kDa bands by western ligand blot assay using ¹²⁵I-IGF-1. On the basis of immunoblotting with bovine IGFBP-2 and -3 antisera, a band for IGFBP-2 was detected at approximately 34 kDa, and a IGFBP-3 band was detected at 38-43 kDa in both adult steers and calves. These results are similar to those observed previous study with Holstein adult steers (Lee et al., 2000).

Furthermore, the 38-43 kDa IGFBP-3 blots were

reduced to a single 36 kDa band after deglycosylation, whereas the 34 kDa IGFBP-2 remained intact in both adult steers and calves (Figure 1b). In previous studies with human (Baxter and Martin, 1986), porcine (Lee et al., 1991) and rat (Yang et al., 1989) sera, the molecular mass of IGFBP-3 has been forward to range from 40 kDa to 45 kDa depending on the number of sites glycosylated. In the present study, the 38-43 kDa IGFBP-3 bands moved to 36.5 kDa band after deglycosylation, however, 38 kDa IGFBP-3 was not completely removed N-glycanase even after it was incubated twice with 4 U N-glycanase. In rat and human sera, IGFBP-3 was shown as a N-glycosylated doublet (Rajaram et al., 1997). However, Wood et al. (1988) reported that the human IGFBP-3 has three potential N-linked glycosylation sites as well as two clusters of serine and threonine residues that can be used for O-linked glycosylation. Thus, our results provide evidence that bovine IGFBP-3 may have other sites probably, O-linked site, that are not deglycosylated with N-glycanase, besides the N-linked glycosylation sites. In general, IGF-1 is associated with IGFBP-3 as well as an acid-labile subunit (ALS) to form a 150 kDa complex in human serum (Baxter et al., 1989). The formation of the complex prolongs its half-life in the blood, thus circulating IGF-1 and IGFBP-3 levels are relatively constant throughout the day in human plasma (Lee et al., 1991). Circulating IGF-1 concentrations in cattle barely changed during a twenty four-h period, despite wide variations in nutrition, metabolism and endocrine systems (Ronge and Blum, 1989). In the present study, plasma IGF-1, IGFBP-3 and other IGFBPs did not show significant variations throughout a twenty four-hr period (Figure 3). This was likely to account for the stability of IGF-1 and IGFBPs levels in blood.

The present study found that concentration the plasma IGF-1 and IGFBP-3 concentrations were increased from 1 m to 17 m. Furthermore, the concentrations of plasma IGF-1 ($r = 0.6801$, $n = 64$, $p < 0.01$) and IGFBP-3 ($r = 0.6321$, $n = 64$, $p < 0.01$) were positively correlated to a gain in liveweight. This was in agreement with the finding in lambs (Roberts et al., 1990) and Holstein seers (Vega et al., 2002; 2004) that plasma IGF-1 was positively correlated with an increase in liveweight. The changes in plasma IGFBP-3, with a gain in liveweight, may be an important control point for increased plasma IGF-1. The IGF-1+IGFBP-3+ALS complex which binds more than 90% of IGF-1 in serum does not cross the capillary endothelium and provides a long-lived, stable reservoir of circulating IGF-1 (Baxter, 1991). Furthermore, plasma IGF-1 clearance was accelerated in situations characterized by a decrease or an absence of serum IGFBP-3 in rats (Davenport et al., 1990). In humans, serum IGFBP-3 was increased until puberty and then continuously decreased with age in adults (Argente et al., 1993; Juul et al., 1995). The pattern showed positive correlation with IGF-1 during various physiological

conditions (Rajaram et al., 1997).

In the present study, the plasma IGFBP-3 concentrations were increased with an increase in liveweight and were positively correlated with IGF-1 concentrations ($r = 0.6191$, $n = 64$, $p < 0.001$). In contrast, the serum IGFBP-2 decreased with age and was inversely correlated to body weight ($r = -0.2919$, $n = 64$, $p < 0.05$), although it did not correlate to plasma IGF-1 concentrations. Furthermore, the proportion of IGFBP-2 of the total IGFBPs in calves was higher than that in adult steers and it was decreased with growth, whereas that of IGFBP-3 was increased with a gain in the liveweight. Serum IGFBP-2 that associates with IGF-1 in smaller complexes can easily cross the capillary endothelium (Bar et al., 1990). The IGFBP-2+IGF-1 complex has mitogenic activity at various tissues. IGFBP-2 enhanced mitogenic activity of IGF-1 in smooth muscle cells (Zapf, 1995).

In conclusion, plasma IGF-1 and IGFBP-3 showed a positive correlation with liveweight but IGFBP-2 showed a negative correlation. The increase in the plasma IGFBP-3 concentration with a gain in liveweight was correlated to the IGF-1 concentration. This suggests that IGFBP-3 has a correlation with growth and may be the control point in modulating the changes in IGF-1 concentration during growth in animals, and IGFBP-2 may play an important role in anabolic action of IGF-1 with a gain in liveweight of young Holstein steers.

ACKNOWLEDGEMENTS

The authors are grateful to the NIDDK program for supplying reagents and procedures for the GH and IGF-I RIA. Appreciation is expressed to Dr. D. Clemmons for providing the rabbit anti-bovine IGFBP-3.

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