Effect of feeding level on serum IGF1 response to GH injection

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Abstract

This study was conducted to further understand the mechanism by which nutrition modulates GH-induced changes in serum IGF1 concentration in cattle. Cows were fed hay only or corn–based concentrates in addition to hay for 8 weeks. At week 8, serum concentrations of IGF1, IGF-binding protein 3 (IGFBP3), and acid-labile subunit (ALS) as well as their mRNA levels in liver were determined immediately before and 7 days after an injection of GH formulated for sustained release. GH injection caused greater increases in both serum IGF1 concentration and liver IGF1 mRNA expression in the cows fed concentrates than in those fed hay. In the cows fed concentrates, the magnitude of the GH-induced increase in serum IGF1 concentration was, however, much greater than that in liver

Introduction

GH is a polypeptide hormone synthesized in the anterior pituitary gland, and is a major regulator of growth and metabolism in many animals. In farm animals, GH stimulates muscle growth and milk production, and inhibits fat deposition (Etherton & Bauman 1998). Based on these effects, injection of exogenous GH has been used in animal industry to increase milk and lean meat production (Etherton & Bauman 1998, Bauman 1999, Foster 1999).

A well-known cellular effect of GH is the enhanced biosynthesis of insulin-like growth factor 1 (IGF1) in the liver (Mathews et al. 1986, Daughaday 2000). Like GH, IGF1 is also an important polypeptide growth factor (Stewart & Rotwein 1996). Most of the circulating IGF1 is produced by the liver (Sjogren et al. 1999, Yakar et al. 1999). Therefore, liver IGF1 was traditionally thought to be the major mediator of the effect of GH on growth (Daughaday 2000). However, recent transgenic mouse studies designed to more definitively determine the role of liver IGF1 in growth have generated conflicting results, with some challenging (Sjogren et al. 1999, Yakar et al. 1999) but others supporting the importance of liver IGF1 in mediating the effect of GH on growth (Liao et al. 2006, Stratikopoulos et al. 2008). Although the role of liver or circulating IGF1 in large animals has not been studied with similar transgenic approaches, many studies have shown that serum IGF1 concentration and liver IGF1 mRNA expression are positively correlated with body gain, feed efficiency, and body protein synthesis rate in multiple domestic farm animals (Hannon et al. 1991, Hayden et al. 1993, Stick et al. 1998), suggesting that liver-derived IGF1 is also important for growth in these animals. Besides GH, a group of IGF-binding proteins, IGFBP1–6, and acid-labile subunit (ALS) serve to regulate circulating IGF1 concentration and action (Boisclair et al. 2001, Firth & Baxter 2002). Under usual physiological conditions, more than 80% of the circulating IGF1 is bound in a complex with IGFBP3 and ALS (Rajaram et al. 1997, Boisclair et al. 2001, Firth & Baxter 2002). This ternary complex is believed to hinder blood IGF1 from crossing the capillary walls to the extravascular tissue, and hence to retain IGF1 in the blood (Rajaram et al. 1997, Boisclair et al. 2001, Firth & Baxter 2002).

It has long been known that the effects of GH on serum IGF1 concentration, growth, or milk production depend on the nutritional status of the animals (McGuire et al. 1992b, Thissen et al. 1994, Etherton & Bauman 1998, Breier 1999).
In cattle, increased feeding of either carbohydrate- or protein-based diet boosted blood IGF1, growth, or milk production response to GH administration compared with ad libitum or restricted feeding (Breier et al. 1988b, McGuire et al. 1992a). Given the apparent role of circulating IGF1 in the control of animal growth and metabolism, the nutrition-dependent IGF1 response to GH likely mediates the nutrition-dependent growth or milk production response to GH. However, the mechanism by which nutrition modulates blood IGF1 response to GH is not completely understood. In this study, we tried to further understand this mechanism in cattle. Specifically, we wanted to know whether the increased nutrition-enhanced serum IGF1 response to GH in cattle is due to increased IGF1 gene expression or increased IGF1 mRNA translation in the liver, or increased retention of IGF1 in the circulation as a result of increased IGFBP3 and ALS concentrations in the blood.

Materials and Methods

Animals, treatments, and sample collection

Twenty non-pregnant, non-lactating Angus crossbred cows were randomly assigned to two groups of 10, which were not different in age or body weight (4.4 ± 0.4 vs 4.6 ± 0.4 years old; 541.0 ± 9.5 vs 538.7 ± 9.5 kg). Both groups of cows were kept in outdoor pens under natural photoperiod and temperature throughout the study, and they had free access to water. The cows in one group, designated the ‘Hay’ group, had ad libitum access to tall fescue hay from the first day (day 0 or d0) to the last day of the experiment (d77). The cows in the other group, designated the ‘Hay + Corn’ group, had ad libitum access to hay from d0 to d7, had a 9:1-kg concentrate meal per cow per day in addition to ad libitum access to hay from d8 to d63, and had ad libitum access to hay from d64 to d77. The concentrate meal was composed of 91.2% corn, 8% soybean meal, and 0.8% urea. This experimental design is illustrated in Fig. 1.

Body weight was recorded weekly at 0900 h (before the concentrate meal). On d0 and d56 of the experiment, each cow was injected s.c. with 500 mg of recombinant bovine GH formulated for slow release (Monsanto Company, St Louis, MO, USA). A blood sample was collected from each cow from the jugular vein via a puncture immediately before and every day after GH administration for seven consecutive days. In addition, weekly blood samples were collected from each cow between the two GH administrations. A liver biopsy (~300 mg) was collected from each cow immediately before and the seventh day after each GH administration (i.e. on d0, d7, d56, and d63). The liver biopsy was performed as described previously (Eleswarapu & Jiang 2005). Blood samples were allowed to clot, and were centrifuged to separate the serum. Serum samples were stored at −20 °C. Liver samples were immediately frozen in liquid nitrogen and stored at −80 °C. Because the tissue sample from the same animal was not sufficient for all the assays, the samples from five randomly selected animals of a group were used for some assays, and the samples from the other five animals from the same group were used for other assays. Neither average age nor average body weight was different between the two groups of five cows.

All animal-related procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RIA

RIA was used to determine serum concentrations of total IGF1 and GH, and was done as described previously (Berry et al. 2003, Wu et al. 2008). An acid–ethanol cryoprecipitation extraction was performed using the serum samples for IGF1 RIA to remove the IGFBPs. The serum samples for GH RIA were not extracted. In the IGF1 RIA, 100 μl of each sample in duplicate were used; the IGF1 standard used was recombinant human IGF1 (GroPep, Adelaide, SA, Australia); the primary antibody used was a mouse anti-human IGF1 antibody previously validated for bovine IGF1 (Berry et al. 2003, Wu et al. 2008). In the GH RIA, 300 μl of each sample in duplicate were used; the GH standard used was a recombinant bovine GH; the primary antibody used was a rabbit anti-ovine GH antibody previously validated for bovine GH (Barnes et al. 1985). The IGF1 RIA had a sensitivity of 23 ng/ml, and an intra-assay coefficient of variation of 9.1%. The GH RIA had a sensitivity of 0.2 ng/ml, and an intra-assay coefficient of variation of 8.1%.

Ribonuclease protection assay

This assay was used to quantify IGF1, IGFBP3, and ALS mRNAs in the liver samples. Total RNA was extracted using TRI Reagent (MRC, Cincinnati, OH, USA), essentially according to the manufacturer's instructions. The quality of the extracted RNA was verified by electrophoresis on agarose gels containing formaldehyde. The probes for the ribonuclease protection assay (RPA) were synthesized from bovine IGF1, IGFBP3, and ALS cDNA.
IGFBP3, ALS, and GAPDH cDNA plasmids in the presence of U-32P-CTP using the Riboprobe Combination Systems kit (Promega). These cDNA plasmids are described in detail in a previous paper (Wu et al. 2008). The RPA was carried out using the RPA II kit (Ambion, Austin, TX, USA) as described previously (Wu et al. 2008). Briefly, 20 μg of total RNA were hybridized with 1 × 10^5 dpm of the probe for IGF1, ALS, or IGFBP3 mRNA, and with 1 × 10^5 dpm of the probe for GAPDH mRNA at 42 °C overnight. The hybridization mixture was then digested with ribonuclease. The undigested probe fragments were resolved through gel electrophoresis, and were subsequently visualized by phosphoimaging. The density of the protected probe fragment representing IGF1, IGFBP3, or ALS mRNA was normalized to that representing GAPDH mRNA in the same RNA sample. The normalized density was compared between treatments.

**Western blot analysis**

This analysis was used to quantify ALS protein in the serum samples, and was carried out as described previously (Wu et al. 2008). Briefly, 0.5 μl of each serum sample was resolved by electrophoresis on a 10% SDS-PAGE. The protein was electrotransferred from the gel onto a nitrocellulose membrane. The primary antibody used was the anti-ALS 1082 antibody (a gift from Dr Yves Boisclair, Cornell University, Ithaca, NY, USA), a rabbit antiserum against a 28-amino acid carboxyl-terminal peptide of bovine ALS (Kim et al. 2006). The secondary antibody used was a HRP-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoblotting signals were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biosystems) under the following conditions: 50–80°C for 2 min, 95°C for 10 min, and 60°C for 1 min. The protein was electro-transferred onto a 12% SDS-PAGE. The protein was electro-transferred onto a nitrocellulose membrane. The membrane was incubated with 1–2.5 × 10^6 dpm/ml of 125I-IGF1 at 4 °C overnight. The membrane was washed to remove unbound 125I-IGF1, and was then exposed to an X-ray film. Equal protein loading and transfer was confirmed by staining the post-transfer gel with Coomassie blue.

**Statistical analysis**

Results are presented as mean ± S.E.M. Multiple means were compared using the Tukey–Kramer Honestly Significant Difference method. Two means were compared using the t-test. Given the small number of animals (n = 5) used in this study, a difference was considered as significant when the P value was < 0.01 and not significant when the P value was > 0.1.

**Results**

**Body weight**

The cows fed hay throughout the 77-d experiment showed no significant changes (P > 0.1) in body weight during the experiment. The cows fed concentrates in addition to hay
from d7 to d63 gained weight during this period, and lost weight from d63 to d77 when they were fed hay only (P<0.05). GH injection on d56 did not change the body weight of the cows fed hay on d63 (P>0.1), but it increased the body weight of the cows fed hay and concentrates on d63 (P<0.1). These data indicate that the effect of exogenous GH on growth in cows might depend on their feeding levels.

**Serum IGF1 concentration**

GH injection on d56 caused continuous increases in serum IGF1 concentration during the 7 days following the injection in both the cows fed hay and the cows fed hay and concentrates (P<0.05, Fig. 2A). The increases were greater in the cows fed hay and concentrates than in those fed hay only (P<0.05, Fig. 2A and B). Serum concentrations of IGF1 on d56 before GH injection were not different between the two groups of cows (P>0.1, Fig. 2A). GH injection also increased IGF1 concentrations from d0 to d7, but these increases were not different between the two groups of cows (data not shown). The IGF1 concentrations appeared to return to their pre-injection levels by the fourteenth day of the injection (i.e. d14 and d70 of the experiment). Overall, additional concentrate feeding enhanced GH-induced increases in serum IGF1 concentration, but it had no effect on basal serum IGF1 concentration.

**Serum GH concentration**

Serum GH concentration was measured to determine whether the greater serum IGF1 response to GH injection in cows fed additional concentrates was due to a greater increase in serum GH concentration. As shown in Fig. 3A, GH injection on d56 increased serum GH concentration from 0.5–2 to 10–20 ng/ml during the 7 days following the injection in both groups of cows (P<0.05), but the increases were not different between the two groups (P>0.1). Pre-injection GH concentration on d56 and post-injection GH concentration on d63 (Fig. 3A) as well as the increases in GH concentration from d56 to d63 (Fig. 3B) were also not different (P>0.1) between the two groups of cows. The GH injection on d0 caused similar increases in serum concentration of GH in both groups of cows (data not shown). The GH concentration appeared to return to its pre-injection levels by the fourteenth day of the injection (i.e. d14 and d70), which was consistent with the fact that the injected GH was formulated for a two-week sustained release (Bauman 1999).

**Liver IGF1 mRNA expression**

Liver IGF1 mRNA expression was quantified to determine whether the GH-induced greater increase in serum IGF1 concentration in cows fed hay and concentrates might be due to a greater increase in IGF1 mRNA expression in the liver. As shown in Fig. 4A and B, GH injection increased liver IGF1 mRNA expression from d56 to d63 in both groups of cows (P<0.1). The GH-induced increase in liver IGF1 mRNA expression was greater in the cows fed additional concentrates than in those fed hay only (P<0.1, Fig. 4B). Liver expression of IGF1 mRNA on d56 or d63 was not different (P>0.1) between the two groups of cows (Fig. 4B).

**Liver IGF1 mRNA translation**

Liver polysomal IGF1 mRNA, the portion of IGF1 mRNA that is actively translated, was quantified to determine the possibility that increased translation of liver IGF1 mRNA contributed to the greater GH-induced increase in serum IGF1 concentration from d56 to d63 in the cows fed concentrates. GH injection increased liver expression of polysomal IGF1 mRNA in both groups of cows from day 56 to d63 (Fig. 5A, left panel, P<0.05), and this increase was...
Nutritional regulation of serum IGF1

The normalized IGF1 was normalized to that of the GAPDH band from the same sample.

average percentage of

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fed hay and concentrates (d56 increased serum IGFBP3 abundance on d63 in the cows to determine this possibility, we measured the serum levels of increased presence of IGFBP3 and ALS protein in the blood. due to more IGF1 being retained in the blood as a result of concentration in cows fed concentrates from d56 to d63 might be greater in the cows fed concentrates and hay than in those fed hay only (Fig. 5A, right panel, P<0.05). However, the average percentage of IGF1 mRNA that was associated with the polysomes on d56 and d63 did not seem to be different between the two groups of cows (Fig. 5B, left panel). The percentage of IGF1 mRNA associated with the polysomes did not appear to change from d56 to d63 in either group of cows (Fig. 5B, right panel). These data indicate that the translational efficiency of IGF1 mRNA in liver was not affected by GH injection or feeding level.

Serum IGFBP3 and ALS protein abundance

The GH-induced greater increase in serum IGF1 concentration in cows fed concentrates from d56 to d63 might be due to more IGF1 being retained in the blood as a result of increased presence of IGFBP3 and ALS protein in the blood. To determine this possibility, we measured the serum levels of IGFBP3 and ALS protein on d56 and d63. GH injection on d56 increased serum IGFBP3 abundance on d63 in the cows fed hay and concentrates (P<0.05, Fig. 6A and B), but not in the cows fed hay only (P>0.1, Fig. 6A and B). The increase in serum IGFBP3 abundance from d56 to d63 in the cows fed additional concentrates was greater than that in the cows fed hay only (P<0.05; Fig. 6B, right panel). GH injection on d56 increased serum ALS levels in both groups of cows (P<0.05, Fig. 6C and D). The increase in serum ALS from d56 to d63 was greater in the cows fed hay and concentrates than in those fed hay only (P<0.05; Fig. 6D, right panel).

Liver IGFBP3 and ALS mRNA expression

Like serum IGF1, most of serum IGFBP3 and ALS are produced by the liver (Scott et al. 1985, Donaghy et al. 1995, Boisclair et al. 2001). Therefore, it is possible that the greater GH-induced increase in serum IGFBP3 and ALS protein levels in the cows fed hay and concentrates was the result of a greater increase in IGFBP3 and ALS mRNA expression in the liver. To determine this possibility, we quantified liver IGFBP3 and ALS mRNA levels on d56 and d63. GH injection on d56 increased liver expression of IGFBP3 mRNA on d63 in the cows fed hay only (P<0.1, Fig. 7A), but it had no effect on liver expression of IGFBP3 mRNA in the cows fed hay and concentrates (P>0.1, Fig. 7B). The d56 to d63 change in liver IGFBP3 mRNA expression was not different between the two groups of cows (P>0.1; Fig. 7B,

Figure 4 Effects of feeding level and GH injection on liver IGF1 mRNA expression. Liver IGF1 mRNA was quantified by ribonuclease protection assay. The GAPDH mRNA was detected simultaneously as an internal control. (A) Representative images of the ribonuclease protection assays. (B) Relative abundance of liver IGF1 mRNA on d56 and d63 and GH-induced changes from d56 to d63. These data were obtained from densitometric analyses of the bands like those in panel A. The density of the IGF1 mRNA band was normalized to that of the GAPDH band from the same sample. The normalized IGF1 mRNA abundance was expressed with an arbitrary unit. Bars labeled with different letters were different (P<0.05, n=5). *P<0.05 versus the other group.

Figure 5 Effects of feeding level and GH injection on liver expression of polysomal IGF1 mRNA. Liver polysomes were isolated by sucrose gradient centrifugation. The abundance of polysome-binding IGF1 mRNA was determined by real-time RT-PCR using GAPDH mRNA as an internal control. (A) Relative abundance of polysomal IGF1 mRNA. Bars labeled with different letters were different (P<0.05, n=5). *P<0.05 versus the other group. (B) Average percentages of total IGF1 mRNA associated with the polysomes. These percentages were obtained by dividing the values given in panel A of this figure by those given in panel B of Fig. 4.
Discussion

This study focused on the mechanism by which feeding level modulates serum IGF1 response to GH. The majority of serum IGF1 is produced by the liver through IGF1 gene expression, which is controlled by GH through the JAK2–STAT5 signaling pathway from the GH receptor (Davey et al. 2001, Woelfle et al. 2003, Eleswarapu et al. 2008). Therefore, one obvious possible mechanism by which GH stimulates a greater increase in serum IGF1 concentration with improved feeding is that increased nutritional intake allows GH to stimulate a greater incremental increase in IGF1 gene expression in the liver. This possibility is supported by our finding that GH injection induced a greater net increase in liver IGF1 mRNA expression only in the cows fed hay and concentrates. However, this study also showed that the GH-stimulated increase in liver IGF1 mRNA expression in the cows fed hay and concentrates was smaller than the corresponding elevation in serum IGF1 concentration. This difference has not been reported before, and suggests that the greater liver IGF1 mRNA response is only partially responsible for the greater serum IGF1 concentration response to GH in cows with increased nutrition.

A second possibility for the greater serum IGF1 concentration response to GH under increased nutritional intake is that GH-induced IGF1 mRNA is more efficiently translated, and therefore, more IGF1 protein is synthesized. This possibility had not been assessed before. This study showed that although there was more IGF1 mRNA associated with the polysomes in the liver of the cows fed both hay and concentrates, the percentage of total IGF1 mRNA associated with the polysomes was not different between the cows fed two different levels of nutrition. These data indicate that increased nutrition enhanced the IGF1 mRNA expression response to GH, but not the translational efficiency of the GH-stimulated IGF1 mRNA. Thus, the data do not support the possibility that the greater serum IGF1 concentration response to GH under increased nutritional intake results from more efficient translation of the GH-induced IGF1 mRNA in the liver.

A third possibility for GH to cause a greater increase in serum IGF1 concentration under increased nutritional intake is that increased nutrition enhances the stability and/or retention of the GH-stimulated IGF1 in the blood. We indirectly determined this possibility by measuring the serum levels of IGFBP3 and ALS, two proteins that form a ternary complex with IGF1 that is believed to block IGF1 from crossing the blood vessels and from being degraded in the blood (Rajaram et al. 1997, Boisclair et al. 2001, Firth & Baxter 2002). Such roles of IGFBP3 and ALS are supported by many studies (Ueki et al. 2000, Boisclair et al. 2001, Modric et al. 2001, Haluzik et al. 2003, Hwa et al. 2006, Domene et al. 2007). Our study showed that GH injection caused a greater increase in both serum IGFBP3 and ALS protein levels in the cows fed additional concentrates than in the cows fed hay only, and that post-GH injection serum concentrations of both IGFBP3 and ALS were greater in the cows fed additional concentrates than in the cows fed hay only. These results support the possibility that under increased nutrition, GH causes a greater increase in serum IGFBP3 and ALS protein, and the increased IGFBP3 and ALS in turn increase the stability and/or retention of IGF1 in the blood. However, in theory, the ternary IGF1/IGFBP3/ALS complex could also function as a reservoir for IGFBP3 and ALS.
ALS in the bloodstream. Hence, an increase in serum concentration of IGF1 could increase that of IGFBP3 and ALS by increasing the retention and/or stability of the latter. This inter-relationship between serum IGF1 and serum IGFBP3 concentrations further increase serum IGF1 concentration. Therefore, it is possible that the greater increases in serum IGF1 concentration or liver IGFBP3 mRNA expression are also controlled by GH, it is not obvious why increased nutrition modulates exogenous GH regulation but not endogenous GH regulation of serum IGF1 concentration or liver IGF1 mRNA expression.

In summary, this study has shown that the overnutrition-enhanced GH stimulation of serum IGF1 concentration in cattle is accompanied by an enhanced GH stimulation of liver IGF1 mRNA, serum IGFBP3, and ALS protein, but not by an enhanced GH stimulation of liver IGFBP3 or ALS mRNA. Based on these results and the fact that the ternary complex of IGF1/IGFBP3/ALS can stabilize and retain each of them in the bloodstream, we propose the following positive

**Figure 7** Effects of feeding level and GH injection on liver expression of IGFBP3 and ALS mRNAs. Liver IGFBP3 and ALS mRNAs on d56 and d63 were quantified by ribonuclease protection assay. Equal RNA loading was controlled by simultaneous detection of GAPDH mRNA. (A and C) Representative images of the ribonuclease protection assays of IGFBP3 and ALS mRNAs respectively. (B and D) Liver IGFBP3 and ALS mRNA abundance on d56 and d63 and GH-induced changes between d56 and d63. These data were obtained by densitometric analysis of the bands like those in panels A and C. Bars labeled with different letters are different (P<0.01, n=5).
feedback mechanism by which overnutrition enhances GH-induced increases in serum IGF1 concentration in cattle: under increased nutrition, GH first induces a greater increase in IGF1 mRNA expression in the liver; this increase directly contributes to the initial increase in serum IGF1 concentration; the resulting increase in serum IGF1 then enhances the retention and/or stability of serum IGFBP3 and ALS through an increased formation of the ternary complex of IGF1/IGFBP3/ALS; and the increased IGFBP3 and ALS help to further increase serum retention and/or stability of IGF1 through the ternary complex.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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