Effect of Dietary Energy and Somatotropin on Components of the Somatotropic Axis in Holstein Heifers*

R. P. Radcliff,^{1,†} M. J. VandeHaar,¹ Y. Kobayashi,¹ B. K. Sharma,¹
H. A. Tucker,¹ and M. C. Lucy²

¹Department of Animal Science, Michigan State University, East Lansing 48824

²Department of Animal Sciences, University of Missouri, Columbia 65211

ABSTRACT

The somatotropic axis, consisting of growth hormone (GH), GH receptor (GHR), insulin-like growth factor (IGF)-I, IGF binding proteins (IGFBP), and IGF receptors, controls growth and mammary development in heifers. Manipulation of the axis with recombinant bovine somatotropin (rbST) improves heifer growth and reduces age at first calving. The effects of rbST are influenced by dietary energy through partially understood mechanisms. The objective was to characterize the somatotropic axis in Holstein heifers fed a diet for either low or high rate of gain and treated with or without rbST. Heifers (120 d of age) were assigned to one of 2 diets to gain either 0.8 kg/d (low, $n = 18$) or 1.2 kg/d (high, $n = 20$). Within each diet, half of the heifers ($n = 9$ for low and $n = 10$ for high) received daily rbST injections (25 $\mu\text{g/kg}$ of body weight). Treatments and diets continued until slaughter (2 mo after puberty). Blood was collected 2 $\times$ per week, and a frequent sampling window was performed 1 d before slaughter. Liver was collected at slaughter. Feeding a high diet or treating with rbST increased serum IGF-I and decreased serum IGFBP-2. The observed changes in serum IGF-I and IGFBP-2 were reflected in their respective liver mRNA amounts. Feeding a high diet decreased serum GH concentrations after rbST injection, but the stimulatory effect of rbST on serum IGF-I was nonetheless greater in high-diet heifers. The differential IGF-I response may be explained by greater GHR 1A in the liver of high-diet heifers. We conclude that a high-gain diet modifies the somatotropic axis in rbST-

treated heifers by decreasing serum GH but increasing serum IGF-I after rbST treatment. Greater IGF-I (indicative of an increased GH response) may be a consequence of greater GHR 1A expression in the liver.

(Key words: growth hormone, IGF-I, heifers)

Abbreviation key: AUC = area under the response curve, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, GH = growth hormone, GHR = growth hormone receptor, IGFBP = insulin-like growth factor binding protein, rbST = recombinant bovine somatotropin, RPA = ribonuclease protection assay.

INTRODUCTION

Prepubertal growth rate and age to puberty affect future productivity of replacement heifers (Gardner et al., 1977; Sejrsen, 1978; Little and Kay, 1979; Radcliff et al., 2000). A high rate of prepubertal growth can be achieved by feeding heifers a diet that is high in energy. However, feeding a high-energy diet increases fat deposition in the mammary gland (Swanson, 1960; Radcliff et al., 1997) and decreases future potential for milk production (Gardner et al., 1977; Sejrsen, 1978; Radcliff et al., 2000). Administration of recombinant bovine somatotropin (rbST) increases growth by repartitioning nutrients toward muscle and away from adipose tissue (Etherton and Bauman, 1998; Breier, 1999). Treating prepubertal heifers with rbST and feeding a high-energy diet may be a method to increase growth and decrease age at puberty without increasing mammary fat deposition and compromising future milk production.

The somatotropic axis, consisting of growth hormone (GH), GH receptor (GHR), IGF-I, IGF binding proteins (IGFBP), and IGF receptors, is essential for growth and mammary development and likely is involved in mediating tissue responses to energy intake and rbST (Breier, 1999). Insulin-like growth factor-I is a potent mitogen produced in response to GH (McGrath et al., 1991; Cohick and Turner, 1998; Liu and LeRoith, 1999; Weber et al., 1999). The majority of IGF-I in circulation comes from the liver (Yakar et al., 1999). Recombinant bST treatment increases liver IGF-I synthesis, and the

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Corresponding author: M. C. Lucy; e-mail: lucym@missouri.edu.

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†Current Address: Department of Animal Science, University of Missouri, Columbia 65211.

subsequent liver IGF-I secretion increases blood IGF-I concentrations (McGuire et al., 1992; Sharma et al., 1994). Changes in blood IGFBP concentrations can modify the actions of IGF-I by sequestering IGF-I in blood and (or) tissues (Clemmons, 1998; McCusker, 1998). In addition to increasing IGF-I synthesis and secretion, rbST increases the activity of metabolic pathways (i.e., gluconeogenic and lipolytic; Etherton and Bauman, 1998) associated with improved growth and lactation (Pocius and Herbein, 1986; Knapp et al., 1992; Bell, 1995). The GHR mediates the actions of rbST, and dietary protein and energy can affect GHR expression (Etherton and Bauman, 1998).

The objective of the present experiment was to determine the effects of dietary energy and rbST on the somatotropin axis and the relationship of the somatotropic axis to body and mammary growth in heifers. Serum and liver were sampled and analyzed for GH, GHR, IGF-I, and (or) IGFBP. Heifer growth and mammary development (reported earlier, Radcliff et al., 1997) were then evaluated relative to the responses that were observed.

MATERIALS AND METHODS

Animals

A detailed description of animal management, diet, and tissue collection was presented in a previous publication (Radcliff et al., 1997). Briefly, prepubertal Holstein heifers (mean BW = 126 ± 3 kg) were maintained at Michigan State University. Heifers were blocked according to BW and assigned to one of 2 dietary treatment groups beginning at 120 d of age. One group ($n = 18$) was fed to gain 0.8 kg of BW/d, (low); whereas the second group ($n = 20$) was fed to gain 1.2 kg of BW/d, (high). Heifers were fed a TMR once daily to achieve the desired weight gain. Composition of the diet was reported in the previous publication (Radcliff et al., 1997). Net energy for maintenance was 1.17 and 1.83 Mcal/kg DM, net energy for growth was 0.57 and 1.20 Mcal/kg DM, and the CP was 16.3 and 19.4% for the low and high diets, respectively. Within each dietary treatment, one-half of the heifers ($n = 9$ for low, $n = 10$ for high) received a daily injection of rbST (25 μ g/kg of BW; Somavubove; Pfizer Animal Health, Kalamazoo, MI) at 0900 h. Control heifers ($n = 9$ for low, $n = 10$ for high) received no injections. Heifers remained on diets and treatment until the time of slaughter (71 ± 1 d after first estrus). Total days on treatment varied from animal to animal because the termination of treatment was based on reproductive maturity. Treatments commenced at approximately 120 d of age and continued for 276 ± 10 d for heifers fed the low diet and 218 ± 9 d for heifers fed the high diet.

Sample Collection

Two days before initiation of treatments, a blood sample was collected from each heifer via jugular venipuncture. A second blood sample was collected 1 wk later, and then blood samples were collected at monthly intervals (30 d) until 90 d after the initiation of treatments. After the first heifer attained 205 kg of BW, blood samples were collected twice a week from all heifers for the purpose of assigning day of puberty (Radcliff et al., 1997). Heifers were fitted with indwelling jugular catheters 2 d before slaughter. Blood samples were collected at 20-min intervals for 6 h on the day before slaughter (0800 to 1400 h; rbST injection at 0900 h). Serum was harvested from blood samples and stored at -20°C until assayed. Heifers were slaughtered by captive bolt stunning and exsanguination. A liver sample (10 g) was collected from each heifer within 20 min after slaughter, snap frozen in liquid nitrogen, and stored at -80°C until RNA isolation. A blood sample (10 mL) was collected from each heifer at the time of slaughter.

RNA Isolation and Northern Blots

The mRNA for IGFBP-2 and -3 in liver on the day of slaughter were quantified at Michigan State University using Northern blots. Total cellular RNA was isolated as described (Sharma et al., 1994). Twenty micrograms of total cellular RNA was fractionated by agarose gel electrophoresis under denaturing conditions and subsequently transferred to nylon membranes (GeneScreen Plus, DuPont NEN Research Products, Boston, MA). The membranes were hybridized with ^{32}P -labeled cDNA probes synthesized from bovine IGFBP-2 and -3 cDNA (gift from R. Renaville, Gembloux Agricultural University, Belgium). Blots were washed and exposed to autoradiography film as described (Sharma et al., 1994).

Ribonuclease Protection Assay

An aliquot of each RNA sample was shipped to the University of Missouri-Columbia. Upon arrival, the integrity of the RNA was determined by electrophoresis of 2.5 μ g of RNA from each sample through a 1% agarose gel in Tris-borate/EDTA buffer (0.09 M Tris-borate, 0.02 M EDTA) with 0.5 μ g/mL of ethidium bromide. All samples had intact 28S and 18S ribosomal RNA and were used for subsequent analyses. Samples were stored at -80°C until ribonuclease protection assay (RPA) for GHR 1A, IGF-I, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The RPA was performed as described (Lucy et al., 1998; Kobayashi et al., 1999a; Kobayashi et al., 1999b) with a commercially available kit (RPA II, Ambion Inc.,

Austin, TX). Twenty micrograms of total cellular RNA was analyzed. Autoradiography was performed with XOMAT-AR film (Eastman Kodak, Rochester, NY) at -80°C for 18 h. Protected fragments were identified by comparing the size of the protected fragment with the undigested probe as well as a radiolabeled 100-bp DNA ladder. The amount of the GHR 1A, IGF-I, and GAPDH mRNA was quantified by scanning densitometry using GP Tools 3.01 (BioPhotonics Corp., Ann Arbor, MI).

Radioimmunoassay and Ligand Blotting

Serum GH concentrations on the day before slaughter (every 20 min for 6 h) were quantified with a double-antibody radioimmunoassay (Gaynor et al., 1995). The intra- and interassay coefficients of variation were 10.1 and 13.9%, respectively. Serum IGF-I concentrations on d -2, 7, 30, 60, and 90 relative to initiation of treatments and d -14, 30, and 72 relative to puberty were also quantified by radioimmunoassay after removal of binding proteins (Sharma et al., 1994). The intraassay coefficient of variation was 11.3%. Serum IGFBP on d 7 and 60 relative to initiation of treatments and d -14, 30, and 72 relative to puberty were analyzed by ligand blotting as described (Sharma et al., 1994).

Statistical Analyses

Serum IGF-I concentrations and serum IGFBP-2 and -3 amounts were subjected to a repeated measures analysis (SAS, 1999). The model included the effects of diet (high vs. low), treatment (with or without rbST), treatment $\times$ diet, animal within treatment $\times$ diet (error term for the preceding effects), day, diet $\times$ day, treatment $\times$ day, and treatment $\times$ diet $\times$ day. The amounts of GHR 1A and IGF-I mRNA (pixel density) were adjusted by dividing the amount of each mRNA by the amount of GAPDH mRNA to correct for minor differences in loading of samples during the gel electrophoresis. The adjusted GHR 1A, IGF-I mRNA, and the IGFBP mRNA were analyzed for the main effects of diet, treatment, and diet $\times$ treatment interactions. Area under the response curve (AUC) for GH from 0800 to 1400 h was calculated using the trapezoidal rule. The greatest serum GH concentration during the 6 h period was designated as peak GH concentration. Both AUC and peak GH concentrations were adjusted by covariance with the serum GH concentration immediately prior to the time when rbST was injected (0900 h) and subjected to ANOVA using mixed models procedures of SAS (SAS, 1999). The model included the effects of diet, treatment, and the diet $\times$ treatment interaction. The covariance structure was diagonal, covariance components were estimated by residual (restricted) maximum likelihood,

Figure 1. Least square means and standard error of means for serum concentrations of insulin-like growth factor-I (IGF-I; A), and the relative amounts of serum insulin-like growth factor binding proteins (IGFBP) -2 (B) and -3 (C) on different days of age and days relative to puberty. Average age at puberty was 313 ± 10 d for heifers fed the low-gain diet without recombinant bovine somatotropin (rbST) injections (Low), 337 ± 10 d for heifers fed the low-gain diet with rbST injections (Low + rbST), 266 ± 10 d for heifers fed the high-gain diet without rbST injections (High), and 269 ± 9 d for heifers fed the high-gain diet with rbST injections (High + rbST; Radcliff et al., 1997). There was an effect of diet, rbST treatment, and diet by rbST treatment on serum IGF-I concentrations ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The high-gain diet decreased serum IGFBP-2 (treatment $\times$ time interaction, $P < 0.01$). Neither diet ($P = 0.13$) nor treatment ($P = 0.15$) affected the serum IGFBP-3. The serum IGFBP-3 increased over time ($P < 0.001$).

and tests were performed using residual degrees of freedom. The data are presented as least square means $\pm$ standard error of means. Statistical significance was defined as $P < 0.05$.

RESULTS

Serum IGF-I, IGFBP-2, and IGFBP-3 Concentrations

There was a diet $\times$ treatment $\times$ time interaction for serum IGF-I concentrations ($P < 0.001$; Figure 1A). As

Figure 2. Least squares means and standard error of means for concentrations of serum growth hormone (GH; A) every 20 min for 6 h. Heifers were fed either a high- or low-gain diet and were either supplemented with recombinant bovine somatotropin (+ rbST) or untreated. Injection of rbST at 0900 h increased mean area under the response curve (AUC; B; $P < 0.001$) and mean peak GH concentrations (C; $P < 0.001$). There was a diet $\times$ treatment interaction for AUC ($P < 0.01$).

expected, rbST increased serum IGF-I concentrations ($P < 0.001$). Diet increased serum IGF-I concentrations as well ($P < 0.001$). The combination of a high-gain diet and rbST treatment resulted in the greatest serum IGF-I concentration ($P < 0.001$). There was an effect of diet ($P < 0.001$) and a treatment $\times$ time interaction ($P < 0.01$) for serum IGFBP-2 (Figure 1B). Heifers fed the high-gain diet had lower serum IGFBP-2 compared with low-gain heifers ($P < 0.001$). The amount of serum IGFBP-2 in heifers treated with rbST was initially similar to that of control heifers ($P = 0.11$), but the amount of IGFBP-2 decreased over time in rbST-treated heifers ($P < 0.01$). There was an effect of time ($P < 0.001$) on serum IGFBP-3 because the serum IGFBP-3 amount increased across the experimental period (Figure 1C). The effects of treatment ($P = 0.13$) and diet ($P = 0.15$) were not significant for serum IGFBP-3.

Serum Concentrations of GH Following rbST Injection

Serum GH concentrations after rbST injection or control are shown in Figure 2A. Regardless of diet, rbST

injection increased the AUC for GH (Figure 2B; $P < 0.001$). There was a treatment $\times$ diet interaction for AUC for GH ($P < 0.01$). The AUC for GH in low-diet heifers was similar to that of high-diet heifers. However, AUC for GH was greater in low-diet heifers injected with rbST than in high-diet heifers injected with rbST ($P < 0.01$). There tended to be a treatment $\times$ diet interaction for peak GH (Figure 1C; $P < 0.10$). Peak GH concentrations were similar in low- and high-diet control heifers. However, peak GH concentrations tended to be greater in low-diet heifers injected with rbST compared with high-diet heifers injected with rbST ($P = 0.08$).

GHR 1A, IGF-I, IGFBP-2, and IGFBP-3 mRNA in the Liver at Slaughter

The GHR 1A mRNA amount was greater in heifers fed the high diet ($P < 0.05$) compared with heifers fed the low diet (Table 1). In contrast, rbST treatment did not affect GHR 1A mRNA amount ($P > 0.10$). There was a tendency ($P < 0.10$) for a treatment $\times$ diet interaction on GHR 1A mRNA. The amount of GHR 1A mRNA in heifers fed the low diet and injected with rbST tended to be less than that of those fed the high diet and injected with rbST.

There was an effect of diet ($P < 0.01$) and treatment ($P < 0.01$) for liver IGF-I mRNA (Table 1). The IGF-I mRNA amount was greater in heifers fed the high diet compared with heifers fed the low diet ($P < 0.01$). In addition, rbST increased the IGF-I mRNA amount ($P < 0.001$). A treatment $\times$ diet interaction was not detected for the mRNA amount.

The amount of mRNA for IGFBP-2 decreased ($P < 0.01$) in heifers fed the high diet compared with low diet and decreased in heifers treated with rbST compared with controls ($P < 0.01$; Table 1). Diet ($P > 0.9$) or rbST ($P > 0.5$) treatment did not affect liver IGFBP-3 mRNA.

Correlations of Serum IGF-I with Growth and Mammary Development

Across all treatment groups, average serum IGF-I (across all days) was correlated positively with total daily BW gain ($r = 0.60$; $P < 0.01$) and mammary parenchymal DNA content at slaughter ($r = 0.44$; $P < 0.01$) (Radcliff et al., 1997). The positive correlation with mammary parenchymal DNA was caused by the rbST treatment. Within the rbST treatment group, serum IGF-I was correlated with BW gain ($r = 0.70$; $P < 0.01$), but serum IGF-I was not correlated with mammary parenchymal DNA. Likewise, serum IGFBP-2 and IGFBP-3 were correlated with BW gain ($r = -0.70$ and

Table 1. Least square means and SEM for liver mRNA (arbitrary densitometric units measured by ribonuclease protection assay or northern blot) in heifers fed either a high- or low-gain diet and supplemented with recombinant bovine somatotropin (+ rbST) or untreated. Type-I error rates (P-values) are presented for diet (D), treatment (T) and the interaction of diet and treatment (D $\times$ T). NS = ($P > 0.10$).

mRNA	Diet and treatment				SEM	<i>P</i> <		
	Low	Low + rbST	High	High + rbST		Diet	T	D $\times$ T
GHR ¹ 1A	467	392	499	569	42	0.02	NS	0.01
IGF-I	208	256	241	307	15	0.008	0.001	NS
IGFBP ² -2	370	140	187	34	46	0.01	0.01	NS
IGFBP-3	189	218	180	232	53	NS	NS	NS

¹GHR = Growth hormone receptor.

²IGFBP = Insulin-like growth factor binding proteins.

$r = 0.40$), but serum IGFBP-2 and IGFBP-3 were not correlated with mammary parenchymal DNA.

DISCUSSION

Heifers fed a high-gain diet and treated with rbST grew faster (Radcliff et al., 1997), and the endocrine changes in response to treatment were as expected. As reported in lactating cows (Sharma et al., 1994), treating with rbST increased liver IGF-I mRNA and blood IGF-I concentrations. Feeding the high-gain diet appeared to amplify the effects of rbST because rbST-treated high-diet heifers had greater blood IGF-I than rbST-treated low-diet heifers. Similar effects of nutrient intake and rbST injections on IGF-I have been reported in young growing Holstein bulls (Smith et al., 2002) and growing beef cattle (Rausch et al., 2002). Greater blood IGF-I in rbST-treated heifers fed the high diet was associated with greater liver IGF-I mRNA. The correlated response between liver IGF-I mRNA and blood IGF-I concentrations illustrates the dependence of blood IGF-I on liver IGF-I synthesis (Yakar et al., 1999). Treatment with rbST decreases relative amounts of IGFBP-2 in cattle (Sharma et al., 1994; Vanderkooi et al., 1995). The magnitude of the decrease in IGFBP-2 caused by rbST was similar for both diets. Serum IGFBP-2 is thought to be inhibitory to IGF-I action (Ronge and Blum, 1989; Ross et al., 1989), and the inhibition of IGFBP-2 by rbST treatment may have increased the biopotency of IGF-I. The changes in serum IGFBP-2 were closely related to the changes in expression of IGFBP-2 mRNA in the liver. Therefore, the liver appears to be a major source of IGFBP-2 whose synthesis may be controlled by GH or IGF-I (Vicini et al., 1991; Cohick et al., 1992; Sharma et al., 1994).

The magnitude of the differences for serum IGF-I and IGFBP-2 in the high-diet-rbST heifers was greater as heifers approached puberty. The greater rbST response may reflect the maturation of the GH/IGF system in peripubertal animals. Badinga et al. (1991) demonstrated greater GHR concentrations in peripubertal

cattle. The improved rbST-response in heifers near puberty (present experiment) suggests that liver GHR concentrations increase in peripubertal animals and supports the previous work of Badinga et al. (1991).

Heifers in this experiment were in positive energy balance, but nutrient intake and growth were greater in heifers fed the high-gain diet compared with heifers fed the low diet. The increase in serum IGF-I in response to rbST was greater in heifers fed the high diet than those fed the low diet (Figure 1). These findings are consistent with studies showing that serum IGF-I response to rbST treatment is greater when cattle are in a more positive energy balance (Vicini et al., 1991; Yung et al., 1996). Feeding Holstein bull calves a high-growth diet increased liver GHR 1A (Smith et al., 2002). Heifers fed the high-gain diet in the current experiment had greater GHR 1A mRNA in the liver as well. Binding of GH to isolated hepatic membranes is highly correlated with GHR 1A mRNA expression (Radcliff et al., 2003). An increase in the amount of liver GHR 1A could increase the capacity for liver GH binding and increase GH-dependent responses like IGF-I synthesis and release. Greater GH binding could also increase the clearance of GH from blood because the liver plays a major role in GH clearance (Lapierre et al., 1992; Lapierre et al., 2000). Greater GH clearance would explain lower serum GH concentrations observed in heifers fed the high-gain diet.

The increase in GHR 1A mRNA in heifers fed the high diet may have been mediated by an increase in insulin concentrations and a subsequent increase in nuclear factors controlling liver-specific genes like GHR 1A. Insulin is required for the normal function of the hepatic somatotrophic axis (Dunger and Cheetham, 1996). Loss of insulin or the reduction in hepatic insulin sensitivity caused a reduction in hepatic GH binding (Baxter et al., 1980) and GHR mRNA amount (Schwartzbauer and Menon, 1998). Insulin infusion increased GHR 1A and IGF-I mRNA in the liver of postpartum dairy cows (Butler et al., 2003). If increased blood insulin concentrations were present in the high-

gain heifers, then greater insulin may have caused the increase in GHR 1A mRNA that we observed.

Daily administration or continuous rbST infusion in lactating cattle increased the liver GHR 1A mRNA amount (Kobayashi et al., 1999b). In the present study, however, the liver GHR 1A mRNA amount did not increase after heifers received daily injections of rbST. This suggests that the daily rbST administration to nonlactating animals does not change GHR 1A mRNA amount, whereas rbST administration to lactating cattle increases GHR 1A mRNA amount. There are several potential explanations for the differences between lactating and nonlactating cattle. First, perhaps endocrine and (or) metabolic changes caused by increased milk production after rbST lead to greater GHR 1A expression. Second, the rbST in the present experiment was administered as a daily injection. In male rats, the amount of GHR1 mRNA (rat equivalent of GHR 1A) was greater when GH was continuously infused, whereas daily GH injection did not alter the GHR1 mRNA amount (Gevers et al., 1996). Therefore, a continuous rbST infusion may be required to increase GHR 1A mRNA in nonlactating cattle. Finally, the GHR mRNA expression in heifers may be at a maximum and refractory to further stimulation by rbST. Badinga et al. (1991) showed that the hepatic binding of GH reaches a peak around the time of puberty in cattle. It may not be possible to increase GHR expression above this relative maximum at puberty.

Rausch et al. (2002) recently reported the results of a similar experiment in beef cattle. They observed lower GH concentrations in ad libitum fed compared with restricted fed heifers. They also reported greater IGF-I, lower IGFBP-2, and greater IGFBP-3 in the blood of rbST-treated heifers. The magnitude of the rbST responses (including growth rate) was greater in older heifers. Many of the rbST responses that we observed were similar to those reported by Rausch et al. (2002). The present experiment provides additional information on liver GHR 1A, IGF-I, IGFBP-2, and IGFBP-3 mRNA that was not reported by Rausch et al. (2002). We also examined the correlation of serum IGF-I with growth and mammary development. Collectively, the 2 experiments [Rausch et al. (2002) and present] provide a consistent picture of the somatotrophic axis in growing heifers fed different diets and supplemented with rbST.

CONCLUSIONS

Feeding a high diet or treating with rbST increased serum IGF-I and decreased serum IGFBP-2. The dynamics of the rbST response was affected by diet because high-diet heifers had lower serum GH concentrations but greater serum IGF-I concentrations after rbST

injection. The differential GH and IGF-I response may be explained by the increase in GHR 1A mRNA in high-diet heifers. The increase in GHR 1A mRNA could theoretically lead to an increase in liver GH binding that would increase GH blood clearance and improve the IGF-I response in high-diet heifers.

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