Effect of nutrition on the GH responsiveness of liver and adipose tissue in dairy cows

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Abstract

Dairy cows enter a period of energy insufficiency after parturition. In liver, this energy deficit leads to reduced expression of the liver-specific GH receptor transcript (GHR1A) and decreased GHR abundance. As a consequence, hepatic processes stimulated by GH, such as IGF-I production, are reduced. In contrast, adipose tissue has been assumed to remain fully GH responsive in early lactation. To determine whether energy insufficiency causes contrasting changes in the GH responsiveness of liver and adipose tissue, six lactating dairy cows were treated for 4 days with saline or bovine GH when adequately fed (AF, 120% of total energy requirement) or underfed (UF, 30% of maintenance energy requirement). AF cows mounted robust GH responses in liver (plasma IGF-I and IGF-I mRNA) and adipose tissue (epinephrine-stimulated release of non-esterified fatty acids

in plasma, IGF-I mRNA, and p85 regulatory subunit of phosphatidylinositol 3-kinase mRNA). Reductions of these responses were seen in the liver and adipose tissue of UF cows and were associated with decreased GHR abundance. Reduced GHR abundance occurred without corresponding reductions of GHR1A transcripts in liver or total GHR transcripts in adipose tissue. In contrast, undernutrition did not alter the abundance of proteins involved in the early post-receptor signaling steps. Thus, a feed restriction reproducing the energy deficit of early lactation depresses GH actions not only in liver but also in adipose tissue. It remains unknown whether a similar reduction of GH action occurs in the adipose tissue of early lactating dairy cows.

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Introduction

Upon parturition, lactating dairy cows enter a period of chronic energy insufficiency (Bell 1995, Block *et al.* 2001). This energy deficit is associated with reduced expression of key metabolic hormones and causes tissue-specific changes in hormone responsiveness (Bell 1995, Bauman 2000, Boisclair *et al.* 2006). For example, dairy cows suffer a reduction of ~70% in plasma insulin-like growth factor-I (IGF-I) during the transition from pregnancy to lactation despite elevated plasma growth hormone (GH; Block *et al.* 2001, Rhoads *et al.* 2004). This fall in plasma IGF-I is thought to reflect decreased GH-stimulated IGF-I transcription in liver as a consequence of GH receptor (GHR) loss (Radcliff *et al.* 2003, Kim *et al.* 2004). Hepatic GHR loss is associated with reduced abundance of the liver-specific GHR1A transcript, whereas the ubiquitously expressed GHR1B and 1C transcripts remain unaffected (Lucy *et al.* 2001, Radcliff *et al.* 2003, Kim *et al.* 2004). These observations

suggest a model whereby the energy deficit of early lactation decreases the activity of the promoter responsible for GHR1A synthesis, leading to a decreased hepatic GHR abundance and IGF-I production (Radcliff *et al.* 2003, Kim *et al.* 2004).

GH actions have also been studied extensively in the adipose tissue of dairy cows (Bauman & Vernon 1993, Bauman 2000). These studies have been performed almost exclusively in post-peak cows in zero or positive net energy balance. Under these conditions, GH attenuates the lipogenic response to insulin and simultaneously amplifies the lipolytic response to β -adrenergic signals (Bauman & Vernon 1993). It is currently thought that these GH actions are maintained or even increased during the energy insufficiency of early lactation when liver actions are reduced (Bauman & Vernon 1993, Butler *et al.* 2003). This assumption is based on the absence of nutritional regulation for the GHR1B and the GHR1C transcripts, which account for all GHR transcripts in adipose tissue (Lucy *et al.* 2001). We are unaware, however,

that this assumption has been verified in adipose tissue of energy-deficient dairy cows by direct measurements of GH-dependent responses and GHR levels.

Our objective was to determine whether the energy deficit typical of early lactating dairy cows caused contrasting changes in the GH responsiveness of liver and adipose tissue and whether tissue-specific changes occurred in the cellular components of GH signaling. Studying effects of negative energy balance on mechanisms regulating GH actions is difficult in early lactation. First, the energy deficit of early lactation is confounded with parturition. Second, data obtained across animals are inherently variable because the metabolic milieu changes at different rates between individuals during the first few weeks of lactation (Ingvarsen *et al.* 2003, Bernabucci *et al.* 2005). As an alternative, we subjected late lactating dairy cows to a severe feed restriction, reproducing the metabolic environment of the early lactating dairy cow. This feed restriction reduced GHR abundance and GH responsiveness not only in the liver but also in the adipose tissue.

Materials and Methods

Animals and design

Six non-pregnant, late lactation cows were used according to the guidance and approval of the Cornell University Institutional Animal Care and Use Committee. Cows were assigned sequentially to a 14-day period of adequate feeding (AF), a 3-day intervening period, and a 14-day period of underfeeding (UF). AF and UF were set to 120% of predicted energy requirements or 30% of maintenance energy requirements (National Research Council 2001) and were achieved by feeding appropriate amounts of a single total mixed ration (1.53 Mcal net energy of lactation and 157 g crude protein per kg dry matter). Daily allowances were distributed into 12 bi-hourly meals with water available at all times.

During the last 10 days of each feeding level, cows were randomly assigned to a single reversal design with 4-day periods

Figure 1 Experimental design. Cows were assigned sequentially to a 14-day period of adequate feeding (AF), a 3-day intervening period, and a 14-day period of underfeeding (UF). During each 14-day period of nutrition, the same experimental procedures were performed as follows. Over the last 10 days of AF and UF periods, cows were randomly assigned to a single reversal design with 4-day periods separated by a 2-day interval. Treatments applied during these 4-day periods were daily i.m. injection of saline or bovine GH (40 mg/day). During each 4-day period, blood samples were collected daily, epinephrine challenges performed on day 3, and liver and adipose tissue biopsies obtained on day 4.

separated by a 2-day interval (Fig. 1). Treatments were daily i.m. injection of saline or bovine GH (40 mg, recombinant bST, lot# 96J-B5128-002, Monsanto, St Louis, MO, USA). During each injection period, daily measurements included feed intake, milk weight, and milk composition by infrared analysis (Dairy One, Ithaca, NY, USA). Body weights were recorded at the start and end of each injection period. Blood samples were obtained immediately before hormone injection at 0900 h. On day 3, epinephrine challenges were performed twice at 1000 and 1400 h, as described by Baumgard *et al.* (2002). At both times, cows were administered an intrajugular bolus of epinephrine (1.4 µg/kg body weight, Anpro Pharmaceutical, Arcadia, CA, USA) and blood samples were withdrawn at fixed times (−45, −40, −30, −20, −10, −5, +2.5, +5, +7.5, +10, +15, +20, +30, +45, +60, +120, +125, and +130 min relative to epinephrine administration). Finally, frequent blood samples were obtained on day 4 of injection every hour between 0900 and 1400 h. Tissues were obtained immediately after frequent sampling as described previously (Houseknecht *et al.* 1995, Rhoads *et al.* 2004). Briefly, liver samples were obtained by percutaneous biopsy with a trocar via a small incision (~1 cm) between the 11th and the 12th rib. Adipose tissue samples were obtained by blunt dissection from an incision (~5 cm) made at the tail-head region. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Analysis of IGF-I and GHR gene expression

Total RNA was extracted by the guanidinium thiocyanate-phenol-chloroform method for ribonuclease protection assays (Chomczynski & Sacchi 1987) and affinity chromatography for real-time PCR assays (RNeasy and on column RNase-free DNase treatment, Qiagen). The quantity and quality of RNA were assessed by absorbance at 260 nm and the RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, CA, USA).

Ribonuclease protection assays (RPAs) were used to measure the abundance of IGF-I and GHR mRNAs in liver and adipose tissue (Kim *et al.* 2004, Rhoads *et al.* 2004). The [³²P]UTP-labeled bovine IGF-I probe yielded a single signal of 200 bp, whereas the [³²P]UTP-labeled bovine GHR1A probe yielded a signal of 312 bp for the GHR1A transcript and a signal of 121 bp corresponding to the sum of GHR1B and GHR1C transcripts (GHR_{1B+1C}). Each RPA was performed in the presence of tenfold molar excess of a low specific activity 18S riboprobe generated from an 18S DNA template (Ambion Inc., Austin, TX, USA). Signals were quantified by phosphorimaging and normalized to the 18S signal.

Real-time SYBR green PCR assays were used to measure transcript abundance of GHR1B, GHR1C, suppressor of cytokine signaling-3 (SOCS3), and p85 regulatory subunit of phosphatidylinositol 3-kinase (p85-PI3KR1) and 18S (Table 1). Briefly, total RNA (2 µg) was reverse-transcribed in a 20 µl volume with 500 ng random primers (Invitrogen) and ImPromII reverse transcriptase (Promega). PCRs were performed in duplicate in a 25 µl volume using Power SYBR Mix (Applied Biosystems, Foster City, CA, USA). Reactions

Table 1 Primers used for real-time PCR assays

Transcript	Sequence ^a
GHRIB	F: GAACCGCGCTCTCTCTCC R: CCCAGAAAAAGCATCACTGG
GHRIC	F: AACTGCTCGAGGCAAGAGAG R: CCCAGAAAAAGCATCACTGG
SOCS3	F: CCCCAGGAGAGCCTATTAC R: ACGGTCTTCCGACAGAGATG
p85-PI3KR1	F: GCTTTTCAGATTCCCAGCAG R: TAGTGGGTTTGGTGTTTG
18S	F: GATCCATTGGAGGGCAAGTCT R: GCAGCAACTTTAATATACGCTATTGG

^aFor each transcript, the sequence of the forward primer (F) and reverse primer (R) are given in the 5' to 3' direction.

contained 500 nM each primer and diluted cDNA (20 ng except 10 ng for 18S). Data were analyzed using a relative standard curve generated using serial twofold dilutions of cDNA prepared and pooled from AF adipose tissue. Unknown sample expression was then determined from the standard curve, adjusted for 18S, and expressed as a fold of AF adipose tissue expression.

Analysis of metabolites and hormones

Plasma glucose, non-esterified fatty acids (NEFAs), β-hydroxybutyrate (BHBA), GH, and insulin were assayed in samples collected at 0900 h on days 3 and 4 of injection. IGF-I was measured in plasma samples collected at hourly intervals on day 4. Metabolites were measured by enzymatic assays and hormones by double antibody RIA using bovine proteins for iodination and standards (recombinant IGF-I, lot GTS-3, Monsanto Co.; rbST, lot 12-77-001, Upjohn Co., Kalamazoo, MI, USA; purified insulin, lot 615-70N-80, Lilly Research Laboratories, Indianapolis, IN, USA). The primary antibodies against IGF-I (rabbit anti-human IGF-I, lot AFP4892898) and GH (rabbit anti-oGH-2) were obtained from the National Hormone and Pituitary Program (Bethesda, MD, USA). The primary antibody for the insulin RIA was purchased from Linco Research Inc. (guinea pig anti-porcine insulin serum, lot 122-845-P, St Charles, MO, USA). The secondary antibodies used were caprine anti-rabbit IgG for the IGF-I RIA (lot 12515, Biotech Source Inc.), ovine anti-rabbit IgG for the GH RIA (kind gift of W R Butler, Cornell University), and goat anti-guinea pig IgG for the insulin RIA (lot GP 2020, Linco Research, Inc). Inter- and intra-assay coefficients of variation for all assays averaged <8 and 9% respectively.

Western immunoblot analysis

Total cellular extracts were prepared by homogenizing tissues (0.5 g for liver and 1 g for adipose tissue) in 2 ml lysis buffer (10 mmol/l Tris (pH 7.6), 10 ml/l Triton X-100, 1 mmol/l

EGTA, 150 mmol/l NaCl, 1 mmol/l Na₃VO₄, 1 mmol/l Na pyrophosphate, 10 mmol/l NaF, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 10 mg/l aprotinin, and 10 mg/l leupeptin). Homogenates were clarified twice by centrifugation (10 000 g for 20 min at 4 °C). An extract enriched in membrane protein was also prepared for adipose tissue, as described by Houseknecht *et al.* (1995). Briefly, 1 g frozen adipose tissue was homogenized in 5 ml sucrose buffer (50 mmol/l Tris (pH 7.6), 250 mmol/l sucrose, 5 mmol/l EGTA, 150 mmol/l NaCl, 1 mmol/l Na₃VO₄, 1 mmol/l Na pyrophosphate, 10 mmol/l NaF, 1 mmol/l PMSF, 10 mg/l aprotinin, and 10 mg/l leupeptin), followed by centrifugal removal of unsolubilized materials (1000 g for 5 min at 4 °C). Membrane proteins were recovered by high-speed centrifugation (100 000 g for 60 min at 4 °C) and resuspended in lysis buffer.

The protein content of extracts was measured by the BCA protein assay (Pierce, Rockford, IL, USA). Fixed protein amounts were electrophoresed on 10% discontinuous SDS-polyacrylamide gels and transferred overnight to nitrocellulose membranes (Schleider and Schuell Inc., Keene, NH, USA). The membranes were blocked for 1 h at room temperature with Tris-buffered saline supplemented with Tween-20 (TBST, 0.05 M Tris (pH 7.4), 0.2 M NaCl, 0.1% Tween 20) containing 5% w/v nonfat-dried skim milk. Membranes were incubated with primary antibodies previously validated with bovine extracts (Kim *et al.* 2004). Antibodies were rabbit anti-human GHR and rabbit anti-mouse JAK2 (Jiang *et al.* 1998, Zhang *et al.* 2001), rabbit anti-mouse STAT5 (#SC-835; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-mouse STAT3 (#SC-482; Santa Cruz Biotechnology), and rabbit anti-rat Sp1 (#SC-59; Santa Cruz Biotechnology). Primary antibodies were diluted in blocking solution (GHR, 1:1000; STAT5, STAT3, Sp1, and JAK2, 1:2000). Following incubation, membranes were washed five times (5 min each) in TBST, then incubated with the secondary antibody (goat anti-rabbit IgG-horseradish peroxidase, KPL, Gaithersburg, MD, USA) at a 1:5000 dilution in blocking solution for 1 h at room temperature. Signals were detected by chemiluminescence (LumiGLO, KPL) and quantified by densitometry using NIH Image software.

Calculations and statistical analyses

Individual net energy balance was estimated as the difference between energy intake and energy expenditure (maintenance and milk energy) essentially as described by Block *et al.* (2001). The energy intake was estimated from intake and chemical composition of feeds (National Research Council 2001). Maintenance requirement was estimated from body weight and milk energy output which was calculated from daily milk yield and composition (National Research Council 2001). The NEFA response over the 0 to +45 min interval was integrated and subtracted from the basal area, defined by the average NEFA concentration between the -45 and 0 min and +120 and +130 min intervals.

The NEFA responses obtained at 1000 and 1400 h were averaged for each cow. The average NEFA responses were normalized by log-transformation before statistical analysis. Hormones, metabolites, and energy-related data were averaged over the last 2 days of each treatment (saline or GH). All data were analyzed by a general linear model accounting for plane of nutrition (Nutrition, AF versus UF), GH (GH, saline versus GH), and their interaction (Nutrition×GH) as fixed effects and animal as the random effect. Unless otherwise mentioned, statistical significance was set at $P<0.05$ for main effects and $P<0.10$ for the Nutrition×GH interaction.

Results

Whole-body GH responses

UF cows were restricted to 11% of the feed consumed by the AF cows (Table 2). Within 3 days of feed restriction, milk yield declined by 70% (Nutrition, $P<0.01$) and remained constant for the next 10 days. Under these steady-state conditions, UF cows reached a net energy deficit of -12.4 Mcal/day, similar to the -11 to -15 Mcal/day range we previously observed during the first week of lactation (Block *et al.* 2001, Leury *et al.* 2003). Relative to AF cows, UF cows had reduced plasma concentration of glucose and insulin and increased plasma concentrations of GH, NEFA, and BHBA (Table 2; $P<0.01$).

GH increased milk yield and the plasma concentrations of glucose and insulin during the AF period, but these responses were lost during the UF period (Table 2; Nutrition×GH, $P<0.01$). GH increased the plasma NEFA concentration to a greater extent during the UF than the AF period (Nutrition×GH, $P<0.01$). When expressed as a fraction

of saline treatment, however, the increase was less during the UF than the AF period (0.36 vs 0.88). Overall, UF cows were similar to energy-deficient, early lactating dairy cows in terms of their metabolic status and inability to mount robust, whole-body GH responses (Vicini *et al.* 1991, Block *et al.* 2001, Rhoads *et al.* 2004).

Hepatic GH responses

Plasma IGF-I is produced predominantly in liver in a GH-dependent manner (Le Roith *et al.* 2001), and therefore was used as an index of hepatic GH responsiveness. Undernutrition caused a 50% reduction in plasma IGF-I (Fig. 2A, $P<0.01$). During the AF period, GH doubled plasma IGF-I but this stimulation was nearly abolished during the UF period (Nutrition×GH, $P<0.01$). The GH-dependent stimulation of hepatic IGF-I mRNA appeared less in UF than AF cows, although this attenuation failed to reach statistical significance (Fig. 2B; nutrition×GH, $P>0.10$).

In energy-deficient, early lactating dairy cows, decreased plasma IGF-I is associated with reduced expression of the liver-specific GHR1A transcript (Radcliff *et al.* 2003, Kim *et al.* 2004). To determine whether this correlation held in underfed late lactating cows, we simultaneously measured the abundance of the GHR1A transcript and the collective abundance of the ubiquitously expressed GHR1B and 1C transcripts (GHR_{1B+1C}). Nutrition had no effect on GHR1A transcript abundance but GH increased its abundance to a similar extent during the AF and UF periods (Fig. 2B; $P<0.05$); neither nutrition nor GH altered GHR_{1B+1C}. Severe feed restriction in late lactation, therefore, failed to reproduce the coincidental reductions in GH-dependent responses and GHR1A seen in early lactating dairy cows.

Table 2 Effect of nutrition and exogenous growth hormone (GH) on energy-related indices and hormones in late lactating dairy cows

Variable	AF ^a		UF ^a		S.E.M.	Significance ^b		
	Saline	GH	Saline	GH		Nutrition	GH	Nutrition×GH
Whole-body energetics								
Dry matter intake (kg/day) ^c	17.9	17.9	2.0	2.0				
Milk yield (kg/day)	18.9	22.6	5.6	7.0	1.0	0.01	0.01	0.01
Energy balance (Mcal/day)	6.1	5.1	-12.4	-13.1	0.6	0.01	NS	NS
Plasma metabolites								
Glucose (mg/dl)	62	66	55	53	1.3	0.01	NS	0.01
NEFA (μM) ^d	92	173	8.4	1098	40	0.01	0.01	0.01
β-Hydroxybutyrate (mg/dl)	5.4	6.4	10.8	11.8	0.6	0.01	0.01	NS
Plasma hormones								
GH (ng/ml)	4.5	6.6	6.5	14.7	1.4	0.01	0.01	0.05
Insulin (ng/ml)	1.58	2.58	0.35	0.29	0.24	0.01	0.05	0.01

^aSix non-pregnant cows were fed amounts corresponding to 120% of predicted energy requirements (AF) or 30% of their maintenance requirements for 14 consecutive days (UF). During each feeding period, saline or GH (40 mg) was administered in a single reversal design with 4-day periods separated by a 2-day interval.
^bSignificance level for the effect of feeding level (Nutrition, AF versus UF), GH (GH, saline versus GH) and their interaction (Nutrition×GH); NS, non-significant, $P>0.05$ for main effects and 0.1 for the interaction.
^cStatistical analysis was not performed because feed intake was set by treatment.
^dNEFA, non-esterified fatty acids.

Figure 2 Effect of nutrition on indicators of GH responsiveness in liver. Six late lactating dairy cows were adequately fed (AF) by providing 120% of predicted energy requirements or underfed (UF) by providing 30% of maintenance requirements. Each plane of nutrition lasted 14 days. During each feeding period, saline or GH (40 mg) was administered in a single reversal design with 4-day periods separated by a 2-day interval. (A) Plasma IGF-I concentration on the fourth day of saline or GH administration. Bars represent the means $\pm$ S.E.M. of plasma IGF-I. The significant effect of nutrition, GH, and their interaction (Nutrition $\times$ GH) are reported. The effect of GH was significant only during the AF period (represented by *). (B) Hepatic IGF-I and GHR gene expression on the fourth day of saline or bST administration. Left: abundance of IGF-I mRNA, GHR1A transcript, and all other GHR transcripts (GHR_{1B+1C}) were measured in 10 μ g total RNA by RPA. Data are from a representative cow. Right: bars represent the means $\pm$ S.E.M. of the IGF-I or GHR1A signal normalized to the 18S signal. The significant effect of GH is reported.

To determine whether this lack of correlation existed also at the protein level, we measured the hepatic GHR abundance by western immunoblotting (Fig. 3). Abundance of the GHR was reduced by 70% during the UF period (GH, $P < 0.01$). GH administration increased hepatic GHR content by 50% during the AF period but this effect was abrogated during the UF period (Nutrition $\times$ GH, $P < 0.08$). Undernutrition did not alter the abundance of the JAK2 kinase responsible for initiating GH signaling or the abundance of the downstream transcription factors STAT3 and STAT5 (Fig. 3). These results indicate that hepatic GHR abundance was reduced during UF, but nevertheless remained sufficient to signal GH-dependent transcriptional responses (i.e., IGF-I and GHR1A mRNA). Undernutrition, however, prevented the translation of these transcriptional events into protein responses.

Adipose tissue GH responses

Catecholamine-dependent NEFA release was stimulated by GH during the AF period, but not during the UF period (Fig. 4A; Nutrition $\times$ GH, $P < 0.05$). To further evaluate the effect of nutrition on adipose tissue, we measured the expression of the GH-stimulated genes IGF-I, p85-PI3KR1, and SOCS3 (Coleman *et al.* 1994, Adams *et al.* 1998, del Rincon *et al.* 2007). GH increased IGF-I and p85-PI3KR1 expression only during the AF period (Fig. 4B and C; Nutrition $\times$ GH, $P < 0.05$). Neither GH nor nutrition altered SOCS3 expression (Fig. 4C). Thus, the positive effects of GH on adipose tissue responses were lost rather than amplified during the period of UF.

As a first step to understand the loss of GH responses in the adipose tissue, we measured the abundance of the GHR (Fig. 5). Both undernutrition and GH reduced GHR protein abundance (Fig. 5A, $P < 0.01$). These effects occurred in the absence of any change in total GHR expression measured by RPA (GHR_{1B+1C}, Fig. 5B). Consistent with the latter, GH and nutrition had no effects on GHR1B and GHR1C transcript abundance measured by real-time PCR (Fig. 5C) or on the ratio of GHR1B to GHR1C ($P > 0.40$, results not shown). Finally, neither nutrition nor GH altered the abundance of the downstream signaling protein JAK2, STAT3, or STAT5 (Fig. 5A). These data show that nutrition and GH regulate GHR abundance in adipose tissue via post-transcriptional mechanisms.

Discussion

Bauman & Vernon (1993) proposed that GH-dependent actions are lost in the liver of energy-deficient, early lactating dairy cows but retained in adipose tissue. In this study, we sought to determine whether an energy deficit leads to contrasting GH responses in liver and adipose tissue and whether any differences could be explained by changes in cellular proteins involved in GH signaling. To do so, we studied late lactation dairy cows first when well-fed and then during a period of severe feed restriction. This sequential design caused shifts in net energy balance similar to those we described previously between late pregnancy and the first week of lactation (Block *et al.* 2001, Leury *et al.* 2003). This similarity also extended to blood variables indicative of energy status (glucose, NEFA, and insulin) and GH function (GH

Figure 3 Effect of plane of nutrition and GH on cellular components of GH signaling in liver. Six late lactating dairy cows were adequately fed (AF) by providing 120% of predicted energy requirements or underfed (UF) by providing 30% of maintenance requirements. Each plane of nutrition lasted 14 days. During each feeding period, saline or GH (40 mg) was administered in a single reversal design with 4-day periods separated by a 2-day interval. Liver biopsies were obtained on the 4th day of saline or GH treatment. Left: liver protein extracts (50 μ g) were analyzed by western immunoblotting for the abundance of the GHR, JAK2, STAT3, and STAT5. The GHR antibody detected signals of $\sim$ 130 and 140 kDa corresponding to mature and immature forms of the receptor (Kim *et al.* 2004). JAK2, STAT3, and STAT5 signals have molecular size of $\sim$ 120, 92, and 95 kDa respectively. The transcription factor Sp1 was also measured as a loading control (doublet of $\sim$ 95 and 105 kDa). Data are from one representative cow. Right: bars represent means $\pm$ S.E.M. of GHR signals. Significant effects of nutrition and the interaction between nutrition and GH (Nutrition $\times$ GH) are reported. The effect of GH was significant only during the AF period (represented by *).

and IGF-I), in terms of both the magnitude of their change and the steady-state concentration reached during the energy-deficient period (either early lactation or UF period). UF cows were also similar to early lactating dairy cows in that they failed to respond to exogenous GH with increased plasma glucose, insulin, and milk output.

Plasma IGF-I is produced predominantly in liver in a GH-dependent manner (Le Roith *et al.* 2001). Early lactating dairy cows have been inferred to suffer from hepatic GH resistance because they have reduced basal and GH-stimulated plasma IGF-I (Vicini *et al.* 1991, Radcliff *et al.* 2003, Kim *et al.* 2004). The simplest explanation for this defect, based on coincidental reduction in hepatic IGF-I and GHR1A mRNA, is that decreased production of the liver-specific GHR1A transcript caused reduced GHR abundance and GH-dependent IGF-I production. This model does not explain our results because UF cows had lower basal and GH-stimulated plasma IGF-I than AF cows but the same GHR1A transcript abundance as AF cows. In agreement with our findings, GHR1A abundance remained unchanged in mid-lactating dairy cows subjected to a less severe feed restriction (Kobayashi *et al.* 2002) and failed to decrease significantly after complete fasting in steers (Wang *et al.* 2003). Thus, it seems that UF alone does not reproduce the reduction in GHR1A seen in early lactating dairy cows.

UF cows had reduced hepatic GHR abundance, in agreement with previous findings in energy-deficient growing cattle and sheep (Breier *et al.* 1988, Bass *et al.* 1991, Newbold *et al.* 1997). Nevertheless, they were able to mount GH-dependent transcriptional responses (i.e., increase in IGF-I and GHR1A mRNA). Therefore, hepatic GHR remains sufficiently abundant in UF cows to mediate the effects of supraphysiological GH on the transcription of IGF-I and GHR genes. However, UF prevented the efficient translation of the GHR transcripts, including that of the GHR1A transcript. Translational regulation of GHR transcripts has not been described before in the liver of feed-restricted cattle, probably because the GHR protein and transcripts have never been measured simultaneously (Newbold *et al.* 1997, Kobayashi *et al.* 2002, Wang *et al.* 2003). In contrast, UF had no effects on the abundance of the intracellular proteins JAK2 and STAT5, which signal the effects of GH on the IGF-I and GHR1A promoters (Herrington & Carter-Su 2001, Woelfle *et al.* 2003, Jiang *et al.* 2007).

Translation is regulated by changes in the abundance and activity of ribosomes (Kimball & Jefferson 1994, Jefferson & Kimball 2001). Both variables are regulated predominantly by the quantity and composition of amino acids reaching the liver. For example, pigs fed a lysine-deficient diet have

Figure 4 Effect of nutrition on indicators of GH responsiveness in adipose tissue. Six late lactating dairy cows were adequately fed (AF) by providing 120% of predicted energy requirements or underfed (UF) by providing 30% of maintenance requirements. Each plane of nutrition lasted 14 days. During each feeding period, saline or GH (40 mg) was administered in a single reversal design with 4-day periods separated by a 2-day interval. (A) Plasma NEFA response to epinephrine challenge. Epinephrine challenges were administered on the third day of saline or GH treatment during each feeding period. Bars represent the untransformed means $\pm$ S.E.M. of the NEFA response (statistical analysis was performed on log-transformed data). The significant interaction between nutrition and GH (Nutrition $\times$ GH, $P < 0.01$) is reported. The effect of GH was significant only during the AF period (represented by *). (B) Abundance of IGF-I mRNA in adipose tissue on the fourth day of saline or GH administration. Left: abundance of IGF-I mRNA was measured in 10 μ g total RNA by RPA. Data are from one representative cow. Right: bars represent the means $\pm$ S.E.M. of the IGF-I signal normalized to the 18S signal. The significant Nutrition $\times$ GH interaction is reported. The effect of GH was significant only during the AF period (represented by *). (C) Abundance of p85-PI3KR1 and SOCS3 analyzed by real-time PCR. Results are expressed relative to expression in adipose tissue of AF cows. Bars represent the means $\pm$ S.E.M. of p85-PI3KR1 and SOCS3. The significant Nutrition $\times$ GH interaction is reported. The effect of GH was significant only during the AF period (shown by *).

reduced plasma IGF-I concentrations in the absence of altered hepatic IGF-I mRNA (Katsumata *et al.* 2002). We are not aware of any data implicating amino acids in regulating GHR translation, but it is notable that substantial translational differences exist among the various GHR transcripts under *in vitro* conditions (Jiang & Lucy 2001). Hepatic amino acid delivery might also explain why translational regulation is seen in UF cows but not in early lactating dairy cows. The 90% feed restriction we used in UF cows must have nearly eliminated portal amino acid delivery. A similar situation, however, does not occur in healthy, early lactating cows because feed intake remains significant on the day of parturition and increases steadily over the first few weeks of lactation (Bell 1995, Block *et al.* 2001). Insulin is a second major regulator of translation and protein synthesis in liver (Kimball *et al.* 1994, Proud *et al.* 2001) and its concentration is also depressed in UF cows. A comparable degree of

hypoinsulinemia in early lactation, however, did not prevent a substantial increase in hepatic GHR between parturition and day 10 of lactation (Kim *et al.* 2004). Collectively, these data suggest that the GHR depression of UF relates to the decreased flux of nutrients reaching the liver rather than hypoinsulinemia.

The GH responsiveness of adipose tissue has been assumed to remain constant or even to increase in underfed dairy cattle (Bauman & Vernon 1993, Butler *et al.* 2003). Because GH increases β -adrenergic responsiveness of ruminant adipose tissue (Bauman & Vernon 1993), we used an epinephrine challenge as an adipose tissue-specific response. As shown by others in lactating dairy cows (McCutcheon & Bauman 1986, Sechen *et al.* 1990), GH increased the effects of epinephrine on plasma NEFA in AF cows, but this response was lost in UF cows. In apparent contradiction with this finding, GH induced a greater increase in plasma NEFA in UF than in

Figure 5 Effect of plane of nutrition and bST on GH signaling in adipose tissue. Six late lactating dairy cows were adequately fed (AF) by providing 120% of predicted energy requirements or underfed (UF) by providing 30% of maintenance requirements. Each plane of nutrition lasted 14 days. During each feeding period, saline or GH (40 mg) was administered in a single reversal design with 4-day periods separated by a 2-day interval. Adipose tissue biopsies were obtained on the fourth day of saline or GH treatment. (A) Cellular components of GH signaling. Left: membrane protein extracts (35 µg) were analyzed by western immunoblotting for the GHR. Cytosolic protein extracts (50 µg) were analyzed by western immunoblotting for the abundance of JAK2, STAT3, STAT5, and Sp1 (loading control). The sizes of the respective signals are given in Fig. 3. Data are from one representative cow. Right: bars represent means ± S.E.M. of the GHR signal. Significant effects of nutrition and GH are reported. (B) Abundance of total GHR mRNA on the fourth day of saline or GH administration. Abundance of all GHR transcripts (GHR_{1B+1C}) was measured in 10 µg total RNA by RPA. The 18S RNA panel is the same as shown in Fig. 4B because the GHR and IGF-I RPA were performed together and resolved on the same gel. Data are from one representative cow. (C) Abundance of GHR1B and GHR1C analyzed by real-time PCR. Results are expressed relative to expression in adipose tissue of AF cows. Bars represent the means ± S.E.M. of GHR1B or GHR1C.

AF cows under basal conditions (Table 2). The reasons for this discrepancy are unknown but could relate to an already maximal stimulation of epinephrine responsiveness by the chronic UF model we used so that no additional differences were detected with bST. Indeed, negative net energy balance is also a strong stimulator of catecholamine-stimulated lipolysis (Ferlay *et al.* 1996, Chilliard *et al.* 1998, Dawson *et al.* 1998). Another possibility is that bST increases the ability of adipose tissue to respond to one or more non-adrenergic lipolytic signals. Obviously, such an effect would not be detected by the epinephrine challenge test.

As a second index of GH action in adipose tissue, we measured the expression of GH-dependent genes. These included IGF-I, which is GH-stimulated in adipose tissue nearly as well as in liver (Coleman *et al.* 1994, Houseknecht *et al.* 2000), and p85-PI3KR1, which mediates the negative

effects of GH on insulin signaling in both skeletal muscle and adipose tissue (Barbour *et al.* 2005, del Rincon *et al.* 2007). GH-stimulated IGF-I and p85-PI3KR1 only in the adipose tissue of AF cows, again consistent with loss of GH responsiveness in adipose tissue of UF cows. In contrast, GH had no effect on SOCS3 expression, including during the AF period when IGF-I and p85-PI3KR1 mRNA responses were observed. Failure to detect GH-dependent stimulation of SOCS3 expression during the AF period likely relates to the ~5-h period separating hormone administration and tissue sampling. Indeed, others have shown that SOCS3 expression increases within 1 h of GH administration but then returns to basal level within the next 3–4 h (Adams *et al.* 1998, Colson *et al.* 2000).

We previously showed that GHR abundance in adipose tissue is reduced by ~36% during the transition from late

pregnancy to early lactation (Rhoads *et al.* 2004). A similar modest reduction in GHR was observed in adipose tissue of UF cows, suggesting that negative energy balance is largely responsible for this effect in energy-deficient, early lactating dairy cows. As in UF liver, the reduction in GHR abundance occurs in the absence of changes in total GHR transcripts. Transcripts belonging to the GHR1B and GHR1C classes account for ~80 and ~20% of all GHR transcripts in the bovine adipose tissue (Lucy *et al.* 1998). Wang *et al.* (2003) reported that one of the GHR1C transcripts was reduced in the liver of fasted steers but no evidence for nutritional regulation has been uncovered for GHR1B in adipose tissue or liver (Lucy *et al.* 2001, Smith *et al.* 2002). Because GHR1C transcripts are translated with greater efficiency than GHR1B transcripts (Jiang & Lucy 2001), it was possible that the reduction in GHR abundance observed in the adipose tissue of UF cows reflected a change in their relative ratio. Our results, however, show that this is not the case because nutrition had no effect on GHR1B and GHR1C transcript abundance or on their ratio. Overall, our results suggest that the reduction in GHR abundance in UF adipose tissue is, as in liver, a consequence of reduced translation. Insulin may be a primary mediator of this response because it increased GHR abundance in adipose tissue of late pregnant and early lactating dairy cows without a change in total GHR expression (Rhoads *et al.* 2004).

In summary, feed restriction recapitulating the energy deficit of early lactation reduced GHR abundance in liver. This reduction, however, appeared to involve an attenuation of translation rather than decreased GHR1A abundance. Therefore, feed restriction cannot be used to uncover the mechanisms whereby negative energy balance causes hepatic GH resistance in early lactating dairy cows. This approach appears appropriate, however, to study how energy insufficiency alters GH action in adipose tissue because feed restriction reduced GHR abundance via impaired translation, as seen in early lactation. This feed restriction model would be particularly useful in determining whether energy insufficiency also restricts the GH responsiveness of adipose tissue by inducing intracellular signaling inhibitors (Gu *et al.* 2003, Flores-Morales *et al.* 2006).

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