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Insulin restores hepatic growth hormone (GH) responsiveness during lactation-induced

negative energy balance in dairy cattle: effects on expression of insulin-like growth

factor-I and GH receptor 1A

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ABSTRACT

Early lactation in dairy cattle is a period of severe negative energy balance (NEB) characterized by reduced blood glucose and insulin concentrations and elevated blood growth hormone (GH) concentrations. The liver is refractory to GH during NEB and this uncoupling of the GH-insulin-like growth factor (IGF) axis results in diminished plasma concentrations of IGF-I. Our objectives were to examine the effects of insulin administration during the immediate postpartum period on plasma IGF-I and GH concentrations and to examine the hepatic expression of total GH receptor (all GH receptor transcripts), GH receptor 1A (GHR 1A) and IGF-I. In addition, we examined adipose tissue for total GH receptor and IGF-I mRNA levels to establish the effects of chronic hyperinsulinemia on an insulin-responsive peripheral tissue. Holstein cows (n = 14) were subjected to either a hyperinsulinemic-euglycemic clamp (insulin; INS) or saline infusion (control; CTL) for 96 hours starting on day 10 postpartum. Insulin was infused intravenously ($1\mu g \cdot kg\ BW^{-1} \cdot h^{-1}$), blood samples were collected hourly, and euglycemia was maintained by infusion of glucose. Insulin concentrations during the infusions were increased 8-fold in INS cows compared with CTL cows (2.33 ± 0.14 vs. 0.27 ± 0.14 ng/ml; $P < 0.001$) while blood glucose concentrations were not different between treatments (45.3 ± 2.2 vs. 42.5 ± 2.2 mg/dl; $P > 0.1$). Plasma IGF-I increased continuously during the insulin infusion, and reached the highest concentrations at the end of the clamp, being almost four-fold higher in INS compared with CTL cows (117 ± 4 vs. 30 ± 4 ng/ml; $P < 0.001$). Hepatic expression of GHR 1A and IGF-I mRNA was low in CTL cows, but was increased 3.6-fold ($P < 0.05$) and 6.3-fold ($P < 0.001$)

respectively in INS cows. By contrast, in adipose tissue the changes in gene expression in response to insulin were reversed with decreases in both total GHR and IGF-I mRNA. The expression of GHR 1A and IGF-I mRNA in liver tissue were correlated in INS ($r = 0.86$; $P < 0.05$), but not CTL cows ($r = 0.43$; $P > 0.1$). Insulin appears to be a key metabolic signal in coupling the GH-IGF axis, thus orchestrating a marked elevation in circulating IGF-I concentrations.

INTRODUCTION

Circulating insulin-like growth factor-I (IGF-I) is produced primarily by the liver in response to growth hormone (GH; Jones & Clemmons 1995) and this relationship forms the basis of the GH-IGF axis. Growth hormone receptors (GHR) are found in many tissues and the liver is the site of greatest abundance (Bornfeldt *et al.* 1989; Brameld *et al.* 1996; Lucy *et al.* 1998; Edens & Talamentes 1998). Expression of the GHR and IGF-I genes in liver are acutely responsive to nutritional status (Bornfeldt *et al.* 1989; Pell *et al.* 1993) and physiological state (Kobayashi *et al.* 1999a). The early lactation period in dairy cattle is characterized by prolonged negative energy balance (NEB) where feed intake is increasing, but energy balance remains negative due to the energetic costs of rising milk production. During this time the liver becomes refractory to GH (Vicini *et al.* 1991) and circulating IGF-I concentrations are dramatically reduced. The decline in circulating IGF-I begins 2 weeks prior to parturition and is paralleled by a decline in plasma insulin. Changes in plasma GH over the same period are opposite to that of IGF-I and insulin (Bell *et al.* 2000). The inability of GH to stimulate hepatic IGF-I production during periods of NEB is termed "GH resistance" (Donaghy & Baxter 1996) and has been documented in many species. It has been well established that hepatic GHR concentration is positively correlated with plasma IGF-I and level of nutrition. Thus GHR concentration may be a major regulatory mechanism for determining activity within the GH-IGF axis (Breier 1999).

The hypoinsulinemia of early lactation is part of a series of coordinated changes that occur around the time of parturition in support of lactation. Low plasma insulin levels reduce glucose uptake by insulin-responsive peripheral tissues (adipose and

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muscle) and facilitate greater uptake of glucose by the mammary gland (Bauman & Elliot 1983); a tissue that is not insulin-responsive. Thus, it is not surprising that genetic advances for milk production have resulted in lower levels of circulating insulin in Holstein cows (Bonczek *et al.* 1988). As insulin is a key signal of metabolic status, we hypothesized that the hypoinsulinemia associated with early lactation was responsible for the specific down-regulation of GHR 1A in liver and that this in turn was responsible for the uncoupling of the GH-IGF axis. Here we describe the effects of a hyperinsulinemic-euglycemic clamp in lactating cows during the NEB of early lactation. An elevation of plasma insulin within the physiological range increased hepatic expression of GHR 1A and IGF-I mRNA resulting in a marked increase in plasma IGF-I levels.

MATERIALS & METHODS

Animals and Treatment

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Beginning on day 8 postpartum, fourteen mature lactating Holstein cows (610 ± 15 kg BW; $\pm$ SE) were provided *ad libitum* access to a total mixed ration formulated to have a net energy for lactation (NE_l) typical for animals in this stage of lactation (1.63 Mcal NE_l/kg). Feed was offered every 2 hours to minimize postprandial variations in nutrient supply and water was freely available at all times. Daily samples of the feed offered were composited on a monthly basis for nutrient analysis (Dairy One Cooperative, Ithaca, NY). Feed refusals were weighed and discarded at noon each day. Cows were milked at 0600 h and 1800 h daily, milk yield

determined, and milk samples were collected for compositional analysis twice weekly (Dairy One Cooperative, Ithaca, NY).

On day 8 postpartum, cows were randomly assigned to either a hyperinsulinemic-euglycemic clamp (insulin; INS) or saline infusion (control; CTL) treatment (n = 7/treatment). For both treatments, three indwelling jugular catheters were inserted (Tygon Microbore Tubing, Norton Performance Plastic, Akron, OH); two catheters on one side were used for infusion of solutions (insulin and glucose or saline) and the catheter on the contralateral side was used to collect blood samples. Baseline measurements (4 blood samples/day) were taken on days 8 and 9, and the treatments were imposed for a 96 h period starting at 1500 h on day 10 and finishing at 1500 h on day 14. As a prophylactic measure, animals were treated with penicillin G procaine during the treatment period (9×10^6 units/day; The Butler Company, Columbus, OH). For the INS group, the target glycemia for each cow ($\pm$ 10%) was the average baseline blood glucose concentration for that individual cow. The insulin infusate was prepared for each cow by dissolving purified bovine pancreatic insulin (I-5500, lot 109H0967, 28.3 USP units/mg; Sigma, St. Louis, MO) in 0.01N HCl, followed by dilution with sterile saline containing plasma (1.25%) from that cow. The insulin solution prepared for each cow was calculated to provide an infusion rate of $1\mu g \cdot kg\ BW^{-1} \cdot h^{-1}$, and was infused via a syringe pump (model SE 400; Vial Medical, Grenoble, France). During the insulin infusion, euglycemia was maintained by infusion of glucose (50% wt/vol dextrose solution; The Butler Company) from sterile bottles at variable rates using a peristaltic pump (Micro/Macro Plum XL; Abbott Laboratories, Morgan Hills, CA). Blood samples were collected hourly during the 96 h infusion period. Blood glucose concentrations

were determined immediately (SureStep Blood Glucose Monitoring System, Lifescan Inc., CA) and the infusion rate of glucose was adjusted if necessary. For CTL animals, sterile saline was infused at a rate of 100 ml · h^{-1} for 96 h, and blood glucose was measured every 4 h.

Energy Balance Determination

Energy balance (EB) was calculated daily from measurements of milk yield and dry matter intake, body weight (weekly measurement), milk-fat percentage (twice-weekly measurement) and the calculated NE_l value of the diet (Beam & Butler 1997). Daily net energy consumed ($NE_{consumed}$) was increased for the animals on the INS treatment by a variable amount depending on the infusion rate of glucose required to maintain euglycemia. The estimated NE_l value of glucose was 3.66 Mcal/kg glucose (Léonard & Block 1997). The estimated glucose NE_l was multiplied by the kg of glucose infused per day and added to the $NE_{consumed}$.

Plasma Measurements

Plasma was collected and stored at -20 ºC four times daily during days 8 and 9 (baseline period) and every 2 hours during the four-day infusion period. The samples were subsequently assayed for insulin, GH, IGF-I, free IGF-I, and nonesterified fatty acids (NEFA). Insulin concentrations were quantified daily on days 8 and 9 and every 2 hours during the 96-hour infusion period by a double-antibody radioimmunoassay (RIA) (Linco Research Inc., St. Louis, MO) as described (McGuire *et al.* 1995a). Plasma GH

and IGF-I were measured daily on days 8 and 9, and every 6 hours during the infusion period. Plasma GH was measured by RIA as described previously (Rosemberg *et al.* 1989) with the exception that the bovine GH for iodination and standards was obtained from Pharmacia Animal Health (Kalamazoo, MI; lot 12, code 77-001). Plasma IGF-I concentrations were quantified by RIA following acid-ethanol extraction as previously described (Rosemberg *et al.* 1989) with the exception that IGF-I for iodination and standards was obtained from Monsanto Co. (St. Louis, MO; lot GTS-2) and the rabbit anti-human IGF-I (AFP4892898) was obtained through the National Hormone and Peptide Program (Dr. A.F. Parlow, scientific director). Free IGF-I (i.e. readily dissociable) was determined for plasma samples taken at 0, 48, and 96 h relative to the start of infusions in a single assay using a commercially available sandwich-type immunoradiometric assay (DSL Inc., Webster, TX) developed for measuring free IGF-I in human serum, but has also been validated for use in bovine fluids (Beg *et al.* 2001). Plasma NEFA concentrations were quantified daily on days 8 and 9, and every 12 h during the infusion period using an enzymatic assay (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Western Ligand Blot of IGF binding proteins

Plasma collected at 0, 48 and 96 h relative to the start of infusions were subjected to western ligand blotting as described by McGuire *et al.* (1995b) to evaluate the temporal changes in IGF-binding proteins (IGFBP) during the infusion period. Plasma proteins were denatured in loading buffer (13.3% SDS, 0.42 M Tris, 0.013% Bromophenol blue, pH 6.5) at 100 ºC for 3 minutes and separated using discontinuous

SDS-PAGE at 175V (double gel unit; Life Technologies Inc., Gaithersburg, MD). Proteins were transferred to a nitrocellulose membrane overnight at 10V using a plate electrode apparatus (BioRad Laboratories Inc., Hercules, CA). Membranes were incubated for 16 h with ^{125}I-IGF-I, washed, and placed on a phosphoimager screen for 48 h. Band intensities were quantified using a Bio-Imaging Analyzer BAS 1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Abundance of the IGFBPs is expressed in arbitrary units. IGFBP-3 and IGFBP-2 were identified on the basis of molecular weight (MW) using the findings from other similar studies on dairy cows that identified these proteins by immunoblot and found them to have a MW and responsiveness to elevated insulin similar to what we observed (McGuire *et al.* 1995b; Mashek *et al.* 2001).

Tissue collection and RNA isolation

Liver and adipose tissues were collected prior to termination of treatments after 96 h of infusion. The liver biopsy was carried out as described by Smith *et al.* (1997). Briefly, a biopsy site between the 11th and 12th ribs was shaved, sanitized with Betadine (Povidone-iodine, 7.5%; Purdue Frederick Co., Norwalk, CT) and 70% ethanol, and anesthetized with lidocaine (lidocaine HCl, 2%; The Butler Company). An incision of approximately 1 cm was made through the skin and the biopsy instrument was used to pierce the intercostal muscles and peritoneum. The liver was located and a 1 to 1.5 g sample was removed. The sample was immediately snap-frozen in liquid nitrogen and stored at –80 °C. The incision site was sutured (Ethicon Inc., Somerville, NJ), and treated topically with Scarlet Oil (Vedco Inc., St. Joseph, MO).

Adipose tissue was collected essentially as described by Houseknecht *et al.* (1995). Briefly, subcutaneous adipose tissue was collected by surgical biopsy from the tailhead region. The area was shaved, and sanitized with Betadine and 70% ethanol. Lidocaine was administered around the biopsy site. A 4 to 6 cm incision was made and subcutaneous adipose tissue removed (2 to 4 g), snap frozen in liquid nitrogen and stored at –80 °C. The incision site was sutured, and treated topically with Scarlet Oil.

Total cellular RNA was isolated using the guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski & Sacchi 1987) with modifications for adipose tissue (Harris *et al.* 1993). The RNA samples were dissolved in sterile H_2O and concentrations determined by spectrophotometry at 260 nm (1 optical density unit = 40µg RNA/ml). Integrity of the RNA was determined by staining a sample of denatured total RNA (15 µg) with ethidium bromide, followed by electrophoresis on a 1.2% agarose-formaldehyde gel. Total RNA extracts with intact 28S and 18S ribosomal RNA bands were used in subsequent real-time RT-PCR assays.

Real-Time RT-PCR

The amount of total GHR (GHR_{tot}), GHR 1A, IGF-I and cyclophilin mRNA in liver tissue were analyzed using Real-Time RT-PCR (reverse transcriptase-polymerase chain reaction). In adipose tissue GHR_{tot}, IGF-I and cyclophilin mRNA were quantified, but GHR 1A mRNA was not measured because GHR 1A is a liver specific transcript (Lucy *et al.* 1998). Total RNA (2.5 µg) was reverse transcribed using SuperScript First Strand Synthesis System for RT-PCR (GibcoBRL, Gaithersburg, MD). Probe and primer sets for bovine GHR_{tot}, GHR 1A, IGF-I and cyclophilin were designed using the Primer

Express Software (Applied Biosystems, Foster City, CA). The primer sequences were as follows:

GHR$_{tot}$ forward, 5'-GGTATGGATCTCTGGCAGCTG-3'; GHR$_{tot}$ reverse, 5'-CTCTGACAAGGAAAGCTGGTGTG-3'; GHR 1A-forward, 5'-CCAGCCTCTGTTTCAGGAGTGT-3'; GHR 1A-reverse, 5'-TGCCACTGCCAAGGTCAAC-3'; IGF-I-forward, 5'-TTGGTGGATGCTCTCCAGTTC-3'; IGF-I-reverse, 5'-GCACTCATCCACGATTCCTGT-3'; cyclophilin-forward, 5'-CACCGTGTTCTTCGACATCG-3'; and cyclophilin-reverse, 5'-ACAGCTCAAAAGAGACGCGG-3'.

The probes for each gene were purchased pre-labeled with a 5' reporter dye [FAM (6-carboxy-fluorescein) or VIC^{TM}] and 3' quencher dye [TAMRA (6-carboxy-tetramethyl-rhodamine)]. Their sequences were as follows: GHR$_{tot}$, 5'-6FAM-TGGCAGGCTCCAGTGATGCTTTTTCT-TAMRA-3'; GHR 1A, 5'-6FAM-TCCATACCTGTAGGACCAAGAGTCCAGCA-TAMRA-3'; IGF-I, 5'-6FAM-CTCGAGCAGTCGGAGGGCGC-TAMRA-3'; and cyclophilin, 5'-VIC-TGTCGACGGCGAGCCCTTGG-TAMRA-3'. The probe for GHR 1A spanned an intron splice site. Twenty-five µl reactions were prepared as recommended by the manufacturer, using 100 nM probe and 500 nM primers and the Taqman Universal PCR Master Mix (Applied Biosystems). Standards (high and low), water (no template control) or samples were added to the reaction mixture. The PCR amplifications and fluorescent detection were performed in triplicate using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). Real-time fluorescence measurements were taken and a

threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline). The C_T value for each sample was proportional to the starting copy number in the PCR reaction (Heid *et al.* 1996). Sequence Detection Software (SDS) (Applied Biosystems) was used to analyze the amplification plots. The C_T for cyclophilin was used to standardize the amount of sample RNA in the reaction, allowing the relative amounts of GHR_{tot}, GHR 1A and IGF-I mRNA to be calculated for each sample.

Statistical Analysis

Statistical analyses were performed using the statistical software package NCSS 2000 (NCSS, Kaysville, UT). The average C_T for the GHR_{tot}, GHR 1A and IGF-I genes were normalized to the average C_T for cyclophilin for each individual animal. The gene expression data was log-transformed to generate equal variances between treatments and subjected to one-way ANOVA. All other parameters were analyzed using repeated measures ANOVA with treatment and time as fixed factors nested within animal. Time included values during the baseline and infusion periods (–48 to 96 h). Values for GH and free IGF-I were log-transformed prior to analysis to generate a normal distribution. When the interaction between treatment and time was significant ($P < 0.05$), pairwise comparisons of individual means were carried out using the Tukey-Kramer test.

RESULTS

Plasma Measurements

Plasma insulin concentrations (mean ± SE) were elevated approximately 8-fold in INS cows over the values in CTL cows during the experimental period (2.33 ± 0.14 vs.

0.27 ± 0.14 ng/ml; treatment P < 0.001; treatment by time, P < 0.001; Figure 1). Blood glucose concentrations, however, were not different between INS and CTL cows over the four-day period (45.3 ± 2.2 vs 42.5 ± 2.2 mg/dl; P > 0.1), and were maintained within ± 10% of baseline values. The glucose infusion began 10 minutes after starting the insulin infusion at an initial infusion rate of 70 g/h. The infusion rate necessary to maintain euglycemia gradually increased over the first 24 hours, and remained relatively constant thereafter at an average rate of 164 ± 1.4 g/h for the remaining 72 hours.

Plasma IGF-I concentrations were increased by insulin treatment (treatment, P < 0.001; treatment by time, P < 0.001; Figure 2). Concentrations were similar during the baseline period (P > 0.05), but started to increase in INS cows after infusions commenced. The IGF-I concentrations were greater by 24 h and continued to rise until the end of the infusion, at which point IGF-I was four-fold higher in INS compared with CTL cows (117 ± 4 vs. 30 ± 4 ng/ml). Plasma free IGF-I concentrations were not different (0.59 ± 0.017 vs. 0.58 ± 0.017 ng/ml; P > 0.05) between treatment groups during the baseline period. During the infusion, free IGF-I was elevated in INS cows compared with CTL cows (treatment by time, P < 0.001), with peak values at 48 h (0.77 ± 0.017 vs. 0.58 ± 0.017 ng/ml; P < 0.05), and remained higher in INS compared with CTL cows at 96 h (0.65 ± 0.017 vs. 0.58 ± 0.017 ng/ml; P < 0.05).

Plasma GH concentrations were not different between the treatments during the baseline period, but declined in both treatments over time (P < 0.001). A trend was observed for GH to be lower in INS cows than in CTL cows (4.7 ± 0.5 vs. 5.9 ± 0.5 ng/ml; treatment, P = 0.11). In INS cows, GH declined to its lowest levels by 36 h after the start of infusion and remained relatively constant thereafter. The CTL cows showed a

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more gradual decline in GH over the infusion period, eventually reaching a final concentration similar to INS cows (Figure 3).

A representative western ligand blot of the temporal profile of IGFBP in plasma is depicted in Figure 4. The abundance of the binding proteins is expressed in arbitrary units. The amount of IGFBP-3 in plasma, observed as 40-44 kDa doublet, was not different between INS and CTL cows at the start of treatment ($P > 0.05$), but was significantly increased during insulin treatment ($P < 0.005$). Plasma levels of IGFBP-3 were increased in INS compared to CTL cows at 48 h (2581 ± 180 vs. 1815 ± 180; $P < 0.07$) and 96 h (2726 ± 180 vs. 1939 ± 180; $P < 0.06$), respectively. The amount of IGFBP-2 (34 kDa band) was similar for INS and CTL cows at 0 h ($P > 0.05$), but was decreased during insulin infusion by 76% (236 ± 87 vs. 964 ± 87; $P < 0.001$) and 83% (132 ± 87 vs. 795 ± 87; ($P < 0.001$) at 48 h and 96 h respectively (treatment, $P < 0.001$; treatment by time, $P < 0.005$). Two additional minor bands were observed to migrate with apparent MW of 29 kDa and 24 kDa. Though the identity of these bands cannot be stated with any certainty, their MW suggests that they might contain IGFBP-1 and IGFBP-4 respectively. Plasma concentration of the 29 kDa binding protein was not different between treatments at 0 h ($P > 0.05$), but was lower in INS cows at 96 h (56 ± 7.7 vs. 99 ± 7.7; treatment by time, $P < 0.001$). Likewise, plasma concentration of the 24 kDa binding protein was similar between INS and CTL cows at 0 h ($P > 0.05$), but was reduced in INS cows at 96 h (32 ± 4.9 vs. 59 ± 4.9; treatment by time, $P < 0.005$).

Plasma NEFA concentrations were similar between treatments during the baseline period ($P > 0.05$), but declined in INS cows during the infusion (treatment, $P < 0.005$; treatment by time, $P < 0.001$). Over the 96 h infusion period, plasma NEFA

concentrations in INS cows averaged 37% of the values found in CTL cows (209 ± 12 vs 563 ± 32 nmol/ml).

Gene Expression

The relative abundance of GHR 1A, IGF-I and GHR_{tot} mRNA in liver tissue are illustrated in Figure 5. Insulin treatment caused a 3.6-fold increase in the level of GHR 1A mRNA ($P < 0.05$). The levels of GHR_{tot} mRNA were not different between treatments ($P > 0.1$). Relative to the CTL cows, insulin treatment increased IGF-I mRNA levels in liver 6.3-fold ($P < 0.001$). Across both treatments levels of GHR 1A and IGF-I mRNA were highly correlated ($r = 0.80$; $P < 0.001$). Within treatments, the correlation was significant in INS cows ($r = 0.86$; $P < 0.05$), but not in CTL cows ($r = 0.43$; $P > 0.1$). The amount of IGF-I mRNA in liver tissue was not correlated with GHR_{tot} mRNA in either treatment.

The amount of GHR_{tot} and IGF-I mRNA were lower in adipose tissue compared to liver ($P < 0.001$). Insulin treatment decreased the abundance of GHR_{tot} and IGF-I mRNA in adipose tissue by 1.8-fold ($P < 0.05$) and 3.4-fold ($P < 0.05$), respectively (Figure 6). Adipose and liver IGF-I mRNA levels were correlated in CTL cows ($r = 0.83$; $P < 0.05$), but were not correlated in INS cows ($r = -0.37$; $P > 0.1$). There was no correlation between hepatic and adipose GHR_{tot} mRNA levels in either treatment. Adipose GHR_{tot} and adipose IGF-I mRNA levels were not correlated in either treatment.

Dry Matter Intake, Milk Production, and Energy Balance

Dry matter intake (DMI), milk production and energy balance were similar between treatments during the baseline period, but there was a reduction in DMI

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(treatment, P < 0.01; treatment by time, P < 0.001) and milk production (treatment, P < 0.01; treatment by time, P < 0.001) in INS cows during the infusion period (Figure 7). The net effect of these changes combined with the NE_l of glucose infused led to greater energy balance in the INS compared with CTL cows (treatment P < 0.005, treatment by time, P < 0.001). Energy balance was 12.9 Mcal/d greater for INS compared to CTL cows for the final three days of the infusion. Dry matter intake at 96 h (i.e., intake consumed over the final 24 h of treatment) was correlated with IGF-I mRNA in INS cows (r = 0.82; P < 0.05), but not in CTL cows (r = 0.22; P > 0.1) and was correlated with GHR 1A mRNA in both INS (r = 0.69; P < 0.1) and CTL cows (r = 0.91; P < 0.01). There was a significant negative relationship between plasma GH and DMI in INS cows (r = -0.86; P < 0.05), but not in CTL cows (r = -0.37; P > 0.1).

DISCUSSION

The most striking observation of this study was the four-fold increase in circulating IGF-I concentration in response to a chronic elevation of plasma insulin during a period of NEB-induced GH resistance. In accordance with the observed increase in plasma IGF-I, hepatic IGF-I mRNA levels were elevated 6.3-fold in INS cows. The increase in IGF-I during insulin infusion was associated with an increase in hepatic GHR 1A, a major GHR transcript in bovine liver tissue. We found GHR 1A was increased 3.6-fold in INS cows, and that GHR 1A levels were correlated with IGF-I mRNA in INS, but not CTL cows (Figure 5). It has been demonstrated previously that hepatic GHR 1A and IGF-I mRNA decline in periparturient cows (Kobayashi *et al.* 1999a), leading to the suggestion that the reduction in GH action, and consequent

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uncoupling of the GH-IGF axis, is due to reduced hepatic GHR 1A (Kobayashi *et al.* 1999b). The results of this study indicate that insulin is an important metabolic signal for the GH-IGF axis, coordinating the parallel increase in liver GHR 1A and IGF-I mRNA resulting in the marked elevation in plasma IGF-I levels. Conversely, both GHR_{tot} and IGF-I mRNA levels in adipose tissue were reduced as a result of the insulin treatment, indicating that insulin induces pleiotropic effects on the GH-IGF axis in different tissues. Two potential mechanistic scenarios exist to explain the observed increase in hepatic IGF-I mRNA: (i) the elevation in insulin had a direct effect on IGF-I gene expression; and (ii) the elevation in insulin stimulated an increase in hepatic GHR expression, allowing GH to act through its cognate receptor to mediate the increased IGF-I output. Information exists to indicate that insulin has stimulatory effects on both of these mechanisms.

Transcription of the IGF-I gene is regulated by multiple factors in various tissues (Adamo 1995). However, the liver is the main source of circulating IGF-I (Yakar *et al.* 1999; Sjögren *et al.* 1999) and hepatic IGF-I synthesis is regulated primarily by GH, nutritional status and insulin (Jones & Clemmons 1995; Thissen *et al.* 1994; Phillips *et al.* 1998). In cultures of primary rat hepatocytes, IGF-I expression was increased by insulin alone (Pao *et al.* 1993; Krishna *et al.* 1996; Boni-Schnetzler *et al.* 1991) and in an additive manner in combination with GH, indicating different modes of action (Krishna *et al.* 1996; Boni-Schnetzler *et al.* 1991). Regions of the rat IGF-I gene downstream of exon 1 are sensitive to nutritional status and *in vitro* transcription was reduced using nuclear extracts from diabetic compared with normal rats (Pao *et al.* 1995). The molecular mechanisms by which insulin acts to increase hepatic IGF-I gene transcription

have recently been elucidated. The ubiquitous transcription factor Sp1 can bind to regions of the IGF-I gene, potentially making a substantial contribution to IGF-I gene expression *in vivo* (Zhu *et al.* 2000). Insulin has profound effects on concentrations of Sp1, both *in vitro* in hepatocytes and *in vivo* in liver tissue of diabetic rats (Pan *et al.* 2001). In addition, a recently identified insulin-responsive binding protein (IRBP) binds to a region of the rat IGF-I gene in close proximity to the Sp1 binding site (Kaytor *et al.* 2001b). Sp1 is known to be involved in heterotypic interactions with many nuclear proteins and is thought to interact directly with IRBP to promote insulin-induced hepatic IGF-I expression (Kaytor *et al.* 2001a). Thus insulin has a direct effect on hepatic IGF-I gene expression by regulating IRBP and Sp1.

Insulin also appears to regulate GHR synthesis in several species. In humans, diabetes is associated with GH resistance because hepatic GHR concentrations are reduced and is thought to be due to portal vein insulinopenia (Hanaire-Broutin *et al.* 1996b). Continuous intraperitoneal insulin infusion (allowing insulin absorption into the portal vein) increased plasma GH binding protein (an indicator of hepatic GHR concentrations) and returned GH responsiveness to normal levels (Hanaire-Broutin *et al.* 1996a). Diabetic human patients with residual beta-cell secretion are capable of producing IGF-I in response to exogenous GH, whereas patients without residual beta-cell secretion are unresponsive to GH (Wurzburger *et al.* 1995). In rats, diabetes leads to reduced liver GH binding and insulin therapy restores GH binding to normal levels (Baxter *et al.* 1980). A recent study has demonstrated that expression of the liver-specific bovine GHR 1A gene is regulated by the liver-enriched transcription factor HNF-4 (Jiang

& Lucy 2001a). Insulin is recognized as a positive modulator of the HNF transcription factor network, including HNF-4 (Duncan *et al.* 1998).

A chronic elevation in plasma insulin concentrations increased plasma IGF-I in dairy cows that were in positive energy balance (i.e., cows that were not GH resistant; McGuire *et al.* 1995b). Although the mechanism for the increased IGF-I was not addressed, a combination of direct insulin action and greater sensitivity of hepatic tissue to GH seems most likely in light of results from other studies. Infusion of insulin and glucose to cows approaching positive energy balance caused an increase in plasma IGF-I compared with cows infused with either saline or glucose (Léonard & Block 1997). GH binding to hepatic microsomal membranes was enhanced in insulin treated cows (Leonard *et al.* 1992), and GH binding was correlated with plasma insulin (Leonard *et al.* 2001). These combined results imply that insulin treatment increases circulating IGF-I, at least in part, by increasing the number of GH receptors in liver. These results are in agreement with our observations of a parallel increase in IGF-I mRNA and GHR 1A following a chronic elevation in plasma insulin.

The miniature *Bos indicus* represents a unique bovine model of Laron dwarfism. The dwarf phenotype is due to low hepatic expression of GHR 1A, and consequent reduction in circulating IGF-I (Liu *et al.* 1999). Circulating insulin levels are very low in these animals and they are unresponsive to exogenous GH in terms of IGF-I release, but GH-responsiveness is restored following insulin therapy, i.e. the hypoinsulinemia appeared to be responsible for the inability of GH to elicit an IGF-I response (Elsasser *et al.* 1990). Thus, we propose that the increase in IGF-I observed in the present study was

not due to the effects of insulin acting alone, but through a combination of insulin effects and insulin-induced restoration of the GH-IGF axis.

Adipose tissue contains high levels of IGF-I mRNA and is GH responsive (Peter *et al.* 1993). Growth hormone promotes lipolysis by antagonizing the anti-lipolytic activities of adenosine and enhancing the lipolytic response to catecholamines (Houseknecht & Bauman 1997; Lanna & Bauman 1999). High levels of circulating GH and low levels of insulin are typical of early lactation, and their respective concentrations facilitate adipose tissue mobilization in support of milk production. We found the expression of both GHR$_{tot}$ and IGF-I mRNA in adipose tissue was reduced following insulin treatment. These results indicate that high insulin levels are inhibitory to adipose GHR expression and thus antagonize the ability of adipose tissue to respond to GH. This is consistent with the observation that genetic selection for increasing milk production is associated with reduced plasma insulin levels and increased circulating GH (Bonczek *et al.* 1988) and that higher producing animals mobilize greater amounts of body reserves in early lactation.

Despite the marked increase in liver GHR 1A mRNA in response to insulin, hepatic GHR$_{tot}$ mRNA (mainly GHR 1A, 1B and 1C) levels were not different between the treatments indicating that transcript(s) other than GHR 1A were downregulated. It is important to note that transcription of the GHR gene is controlled by multiple different promoters that could respond differently to the same signal. Although GHR 1B appears to be unregulated, it's worthy to note that the promoter region of GHR 1B contains both negative and positive regulatory elements influencing transcriptional activity (Jiang *et al.* 2000). In addition, the various transcripts are controlled by translational mechanisms,

with GHR 1B being poorly translated and the other transcripts generally having high translational efficiency *in vitro* (Jiang & Lucy 2001b). The physiological importance of changes in GHR transcripts other than GHR 1A in liver tissue are not well defined, whereas the importance of hepatic GHR 1A expression in relation to the functionality of the GH-IGF axis has been clearly demonstrated (Kobayashi *et al.* 1999a; Smith *et al.* 2002). Thus it's possible that insulin increased GHR 1A transcription in liver via stimulation of liver enriched transcription factors (including HNF-4), and downregulated other transcripts in both tissues by as yet undetermined mechanisms.

Pituitary GH release is primarily determined by hypothalamic GH-releasing hormone and somatostatin, but is also stimulated by ghrelin, and inhibited by plasma IGF-I via a negative feedback loop (Berelowitz *et al.* 1981; Kojima *et al.* 1999). GH release should be suppressed in the INS cows due to elevated plasma IGF-I. We observed a decrease in GH levels in the INS cows after approximately 30 hours that was maintained for the remainder of the infusion but values were not different from saline infused cows at the end of the infusion period (Figure 3). This may be ascribed to the reduced intake in the INS cows, and its attendant effects on clearance of GH from plasma and ghrelin release. The metabolic clearance rate of GH in ruminants is slower during periods of chronic feed restriction (Trenkle 1976; Lapierre *et al.* 1992). Conflicting reports exist relating the role of plasma insulin in regulating circulating ghrelin (Caixas *et al.* 2002; Saad *et al.* 2002), but ghrelin is clearly elevated during starvation in rats (Tschop *et al.* 2000) and in cows prior to feeding (Hayashida *et al.* 2001). It is clear that IGF-I acts as a negative feedback regulator of GH release, but this effect was confounded in the present study by the effect of insulin administration to depress DMI.

Insulin infusion altered IGFBP and increased free IGF-I concentrations in plasma. The marked reduction in IGFBP-2 is in agreement with numerous reports showing that IGFBP-2 is inhibited by insulin (Brismar *et al.* 1988; Boni-Schnetzler *et al.* 1990; McGuire *et al.* 1995b; Scharf *et al.* 1996; Mashek *et al.* 2001). In contrast to previous reports (McGuire *et al.* 1995b; Mashek *et al.* 2001), we observed IGFBP-3 to be significantly higher in the insulin treated cows. This is consistent with the identification of an insulin response element in the rat IGFBP-3 gene (Villafuerte *et al.* 1997). Though the plasma concentrations of the 29 kDa (potentially containing IGFBP-1) and 24 kDa (potentially containing IGFBP-4) binding proteins were low in all cows, significant reductions in both were observed with insulin treatment. The net effect of the insulin treatment was to reduce the abundance of the lower MW binding protein complexes and increase the abundance of IGFBP-3, thus increasing the proportion of IGF-I circulating in the 150 kDa ternary complex. As the half-life of the ternary complex is much longer (~12 h) than the half-lives of the lower MW complexes (30 – 90 minutes), a shift from low MW binding proteins to IGFBP-3 reduces the clearance rate of IGF-I (Thissen *et al.* 1994; Jones & Clemmons 1995). Thus, the shift in the relative proportions of IGFBP-2 and IGFBP-3 observed in this study represents another mechanism by which insulin regulates plasma IGF-I concentrations. Though free IGF-I is rapidly cleared from the circulation (Jones & Clemmons 1995), we observed an increase in free IGF-I during hyperinsulinemia as previously reported in humans (Frystyk *et al.* 1997). This reflects the effect of insulin to reduce the presence of the low MW complexes coincident with increased transcription and secretion of IGF-I. The physiological significance of increasing the concentration of free IGF-I merits further study.

In summary, we found that an elevation in plasma insulin within the physiological range during NEB increased plasma IGF-I by approximately four-fold. The increase in plasma IGF-I was associated with an increase in hepatic GHR 1A and IGF-I mRNA. To our knowledge, this is the first report to demonstrate that GHR 1A is responsive to circulating insulin levels. As GHR 1A is a major GHR transcript in liver (Jiang & Lucy 2001b), it is apparent that insulin plays a key role in regulating hepatic GHR concentration. It is likely that insulin itself also contributed to the observed increase in IGF-I by direct actions on the IGF-I gene. Based on our current observations of the effects of hyperinsulinemia during NEB, we propose the model outlined in Figure 8 to illustrate the interaction between plasma insulin and GH in influencing circulating IGF-I concentration and adipose tissue mobilization. Further work is necessary to confirm that experimentally induced hypoinsulinemia would result in the proposed outcome in liver and adipose tissue. However, it has already been demonstrated that a rapid drop in insulin in periparturient cows is associated with a decrease in hepatic GHR 1A expression (Kobayashi *et al.* 1999a). We propose that the hypoinsulinemia observed in periparturient dairy cows plays a role in reduced hepatic GHR 1A expression, resulting in uncoupling of the GH-IGF axis. Restoration of plasma insulin to a certain threshold level may be an important component of regenerating a functional GH-IGF axis in early lactation cows and in other negative energy balance situations.

ACKNOWLEDGEMENTS

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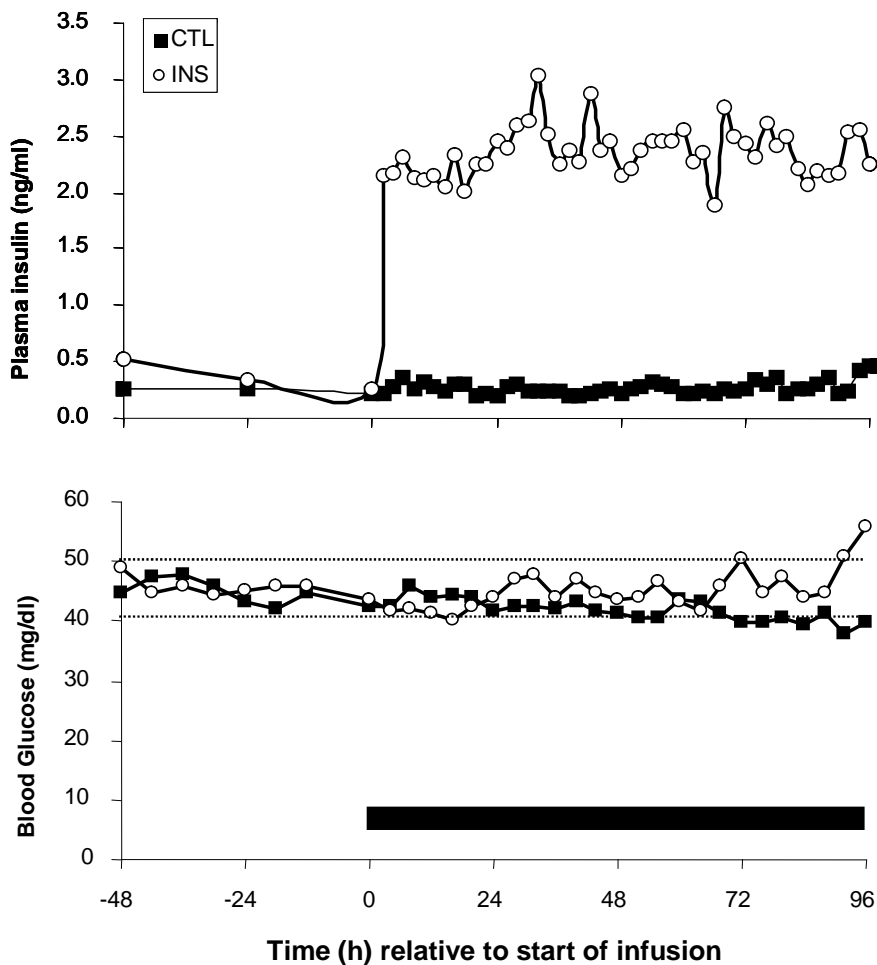
Figure 1. Mean plasma insulin and blood glucose concentration during the 48-h baseline and 96-h infusion periods. *Upper panel:* in INS cows, insulin infusion commenced at time 0 and continued for 96-hours (solid bar), whereas CTL cows were infused with saline. Plasma insulin levels were measured at –48 and –24 hours, and every 2 hours during the infusion period in both treatment groups. Significant treatment ($P < 0.001$) and treatment by time ($P < 0.001$) effects were observed (pooled SEM = 0.14 ng/ml). *Lower panel:* blood glucose was measured every 6 hours during the 48 hour baseline period, and hourly during the infusion period. Glycemia was maintained within ± 10% of baseline values (dotted lines).   Blood glucose levels were not different between treatments (pooled SEM = 2.2 mg/dl; $P > 0.1$).

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Figure 2. Mean plasma IGF-I in INS and CTL cows during the 48-h baseline and 96-h infusion periods. Plasma IGF-I levels were measured at –48 and –24 hours, and every 6 hours during the infusion period for both treatments (pooled SEM = 4.9 ng/ml). Significant treatment (P < 0.001) and treatment by time (P < 0.001) effects were observed. The solid black bar represents the infusion period.
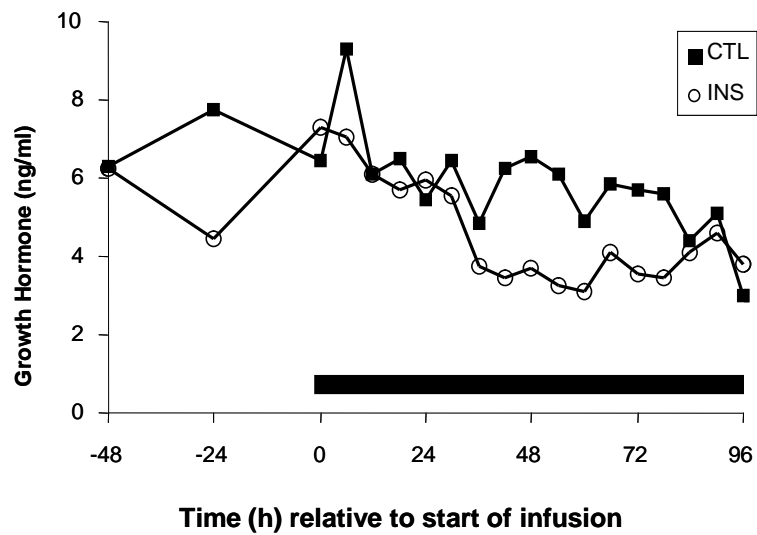
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Figure 3. Plasma GH concentrations during the baseline and infusion periods in INS and CTL cows. Plasma GH was measured at –48 and at –24 hours, and every 6 hours during the 96-hour infusion period (solid bar). The effects of treatment ($P > 0.1$) and treatment by time ($P > 0.1$) were not significant (pooled SEM = 0.54 ng/ml).
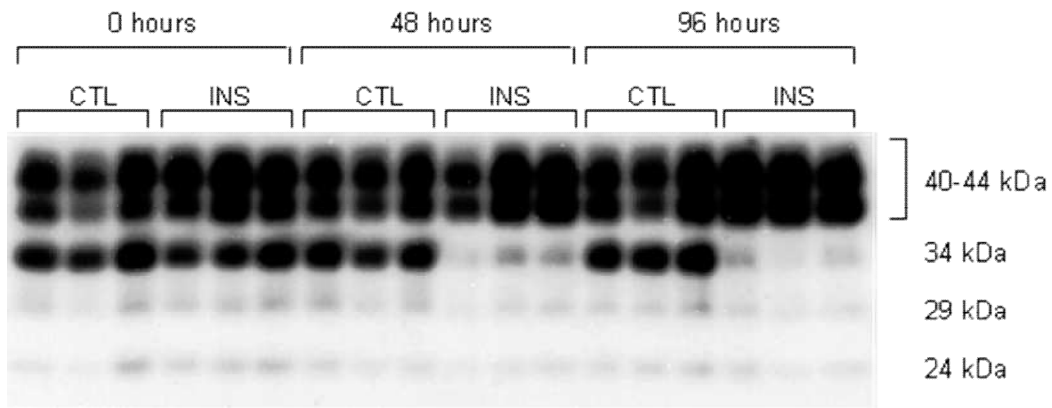
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Figure 4. Effect of treatments on plasma IGFBP at 0 h, 48 h and 96 h relative to the start of infusion on 3 representative cows per treatment. Plasma samples were analyzed by western ligand blot with ^{125}I-IGF-I. Molecular masses (kDa) are indicated. See text for details.
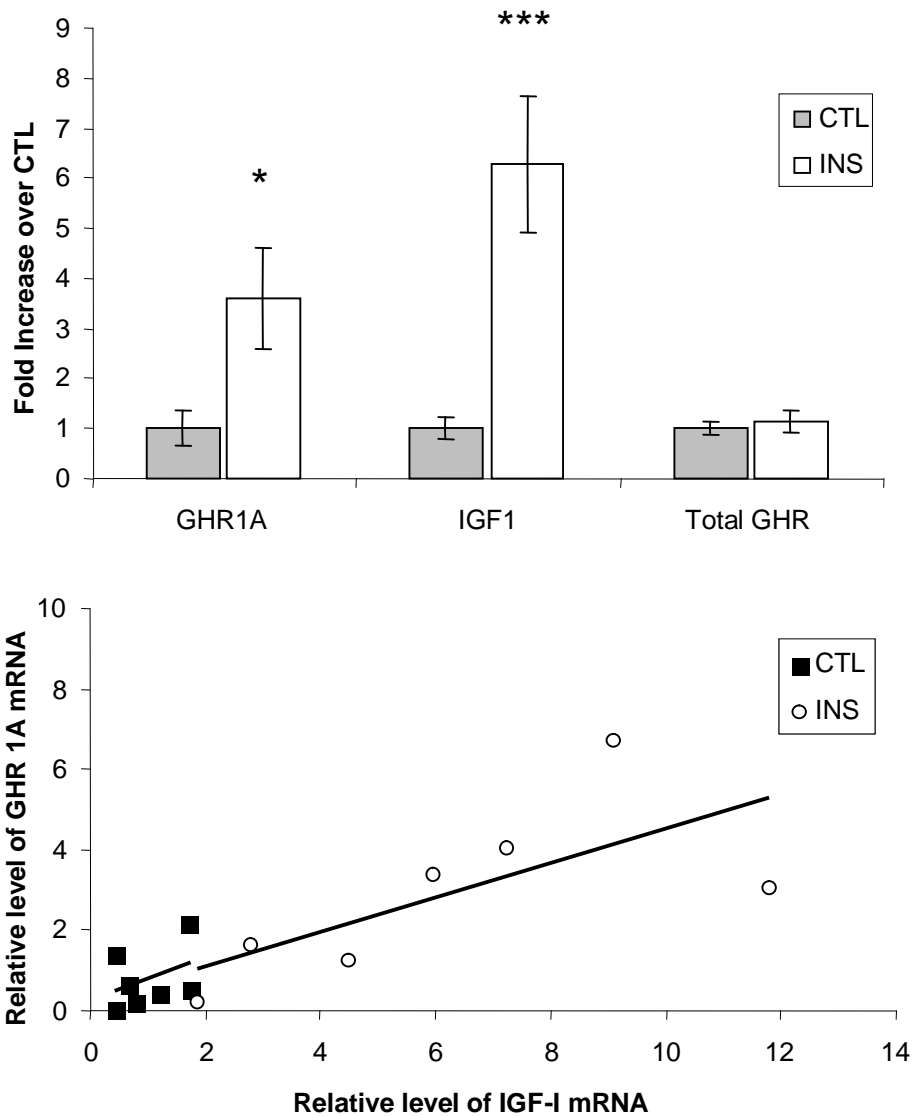
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Figure 5. Relative levels of liver GHR-1A, IGF-I and total GHR mRNA and the correlation within each treatment for GHR 1A and IGF-I mRNA. *Upper panel:* RNA was extracted from liver, and subjected to real-time RT-PCR using probes for total GHR, GHR 1A, IGF-I, and cyclophilin. The total GHR, GHR 1A and IGF-I mRNA data were corrected with cyclophilin, and the value for CTL cows set at 1 for each gene. Expression of the cyclophilin gene was not different between treatments ($P > 0.1$). Values are means ± SE. Significance of difference from CTL is indicated by asterisks: *$P < 0.05$; ***$P < 0.001$. *Lower panel:* A positive correlation was observed between GHR 1A and IGF-I mRNA in INS cows ($r = 0.86$; $P < 0.05$), but not in CTL cows ($r = 0.43$; $P > 0.1$). The values used in the correlation analysis were unadjusted data points.
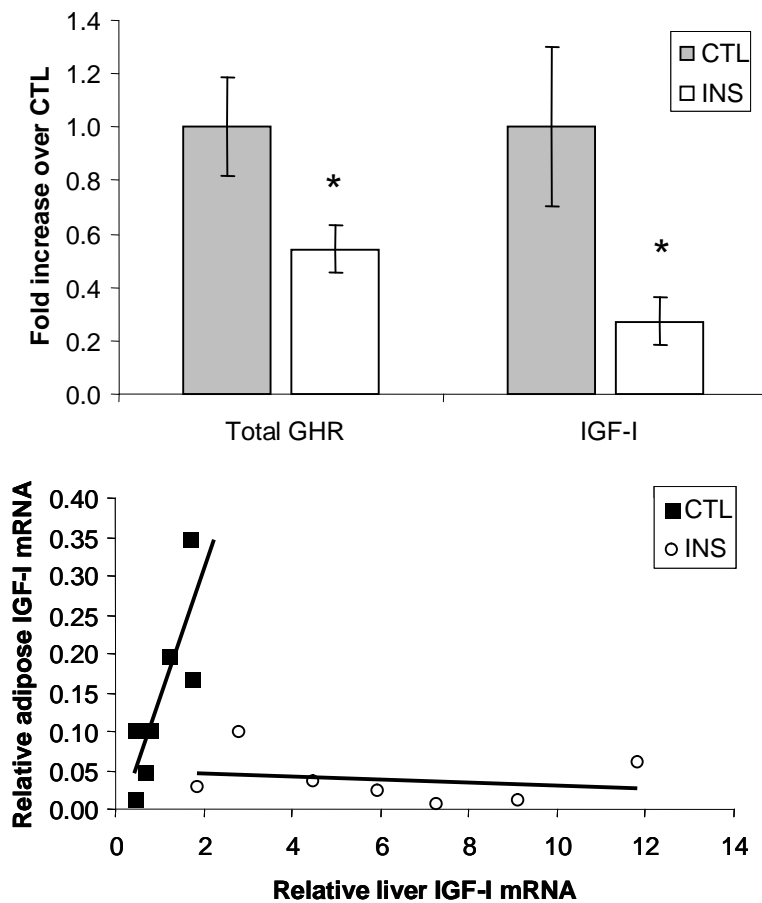
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Figure 6. Relative level of total GHR and IGF-I mRNA in subcutaneous adipose tissue taken prior to terminating the infusions and the relationship between hepatic and adipose IGF-I mRNA levels. *Upper panel:* RNA was extracted from adipose tissue, and subjected to real-time RT-PCR using probes for total GHR, IGF-I and cyclophilin. The total GHR and IGF-I mRNA data was corrected with cyclophilin, and the value for total GHR mRNA and IGF-I mRNA in CTL cows set at 1. Expression of the cyclophilin gene was not different between treatments ($P > 0.1$). Values are means ± SE. Significance of difference from CTL is indicated by an asterisk: *$P < 0.05$. *Lower panel:* A positive correlation was observed between adipose and liver IGF-I mRNA in CTL cows ($r = 0.83$; $P < 0.05$), but not in INS cows ($r = 0.37$; $P > 0.1$). The values used in the correlation analysis were unadjusted data points.
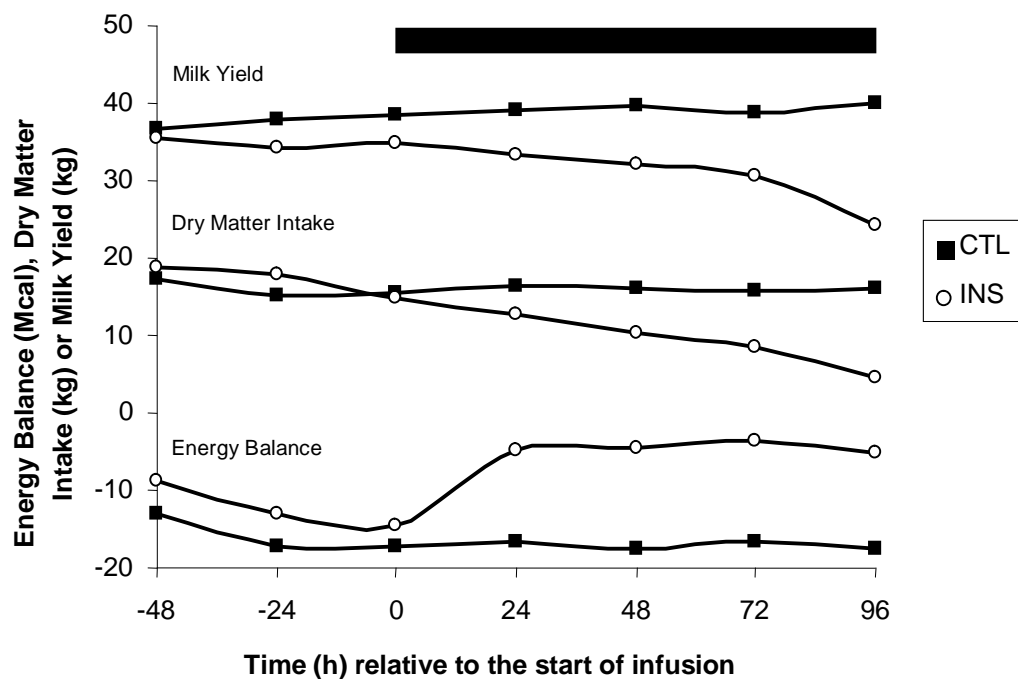
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Figure 7. Milk yield, dry matter intake and energy balance over the 48-hour baseline and 96-hour infusion periods. The solid bar represents the infusion period. Milk yield and dry matter intake decreased during the infusion period for INS cows (treatment, $P < 0.01$; treatment by time, $P < 0.001$). Energy balance was improved in the INS compared with CTL cows during the infusion period (treatment, $P < 0.005$; treatment by time, $P < 0.001$).
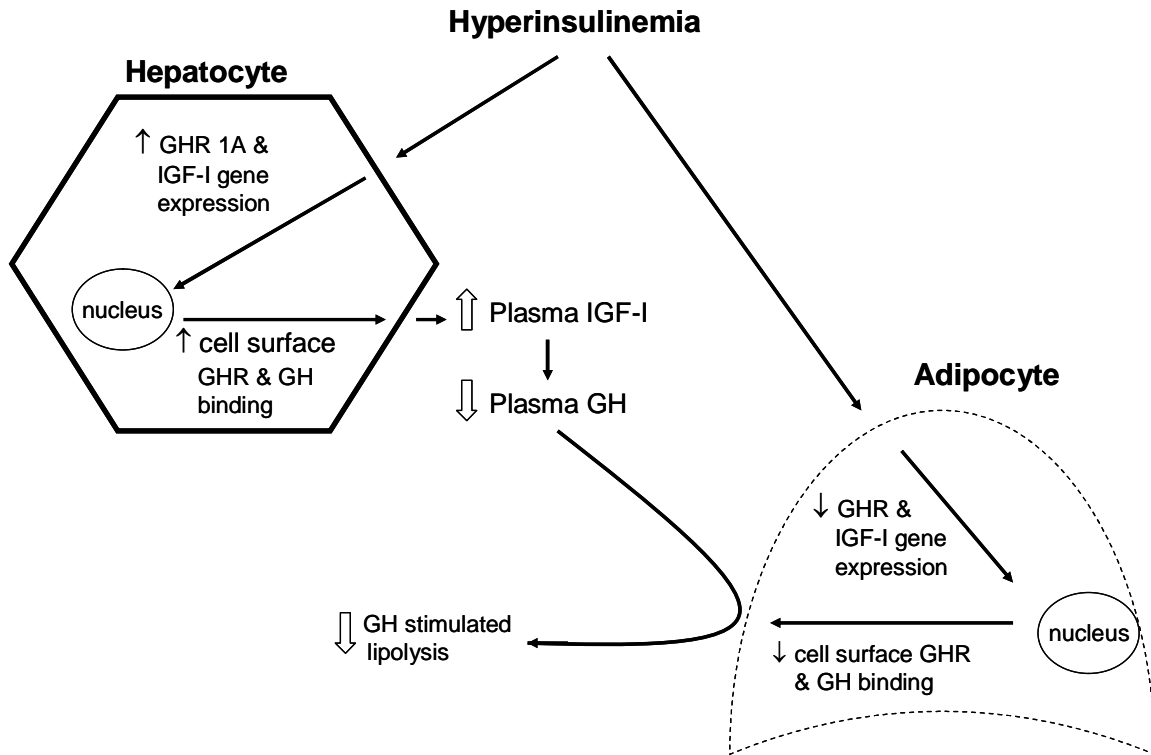
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Figure 8. Model of the interaction between plasma insulin and the GH-IGF axis in liver and adipose tissue. Insulin stimulates hepatic expression of both GHR and IGF-I, resulting in increased plasma IGF-I. High IGF-I acts to decrease plasma GH. In adipose tissue, high plasma insulin is inhibitory to both GHR and IGF-I expression. The reduced adipocyte GHR concentration coincident with low plasma GH results in reduced GH-stimulated adipose tissue mobilization. During situations of low plasma insulin, including periods of NEB, the opposite occurs. Hepatic expression of GHR and IGF-I is reduced, resulting in reduced plasma IGF-I and increased plasma GH. In adipose tissue, GHR expression is up-regulated during hypoinsulinemia. The increased adipocyte GHR concentration and high plasma GH levels results in increased adipose tissue mobilization.

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