

## Insulin Increases the Abundance of the Growth Hormone Receptor in Liver and Adipose Tissue of Periparturient Dairy Cows<sup>1</sup>

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**ABSTRACT** After parturition, increased growth hormone (GH) secretion is important to preserve the metabolic homeostasis of energy-deficient dairy cows. Elevated plasma GH promotes lipid mobilization from adipose tissue, but paradoxically, is associated with depressed concentration of insulin-like growth factor-I (IGF-I), a growth factor produced in a GH-dependent fashion in liver. Primary factors regulating GH responses of liver and adipose tissue are poorly understood in periparturient dairy cows. Consistent with insulin being such a factor, its plasma concentration declined concomitantly with net energy balance (EB) and with plasma IGF-I in a group of 9 periparturient dairy cows. To test the role of insulin in regulating cellular determinants of GH responsiveness, hyperinsulinemic-euglycemic clamps were performed on 6 dairy cows in late pregnancy (28 d prepartum) before the reductions in EB, insulin, and IGF-I were initiated, and when they were completed in early lactation (10 d postpartum). Infusion of insulin nearly doubled the plasma concentration of IGF-I ( $P < 0.001$ ) and hepatic levels of IGF-I mRNA during both states ( $P < 0.05$ ). In liver, these responses were associated with increased abundance of the GH receptor protein (GHR;  $P < 0.05$ ), whereas the abundance of intracellular mediators of GH actions (JAK2, STAT5, or STAT3) remained unaffected. Insulin also doubled GHR abundance in adipose tissue ( $P < 0.01$ ), indicating that this effect is not liver specific. These results raise the possibility that insulin regulates the efficiency of GH signaling in liver and adipose tissue of dairy cows by acting as a rheostat of GHR synthesis. *J. Nutr.* 134: 1020–1027, 2004.

**KEY WORDS:** • pregnancy • lactation • hyperinsulinemic-euglycemic clamp • insulin-like growth factor-I

In high-yielding dairy cows, the onset of lactation increases the total energy requirements by ~4 fold, reflecting mainly the nutrient needs of the mammary gland for milk synthesis (1,2). Because the hyperphagia required to meet those demands develops slowly, shortfalls are met by mobilization of endogenous reserves and by shifting the pattern of nutrients used by nonmammary tissues (1,2). These metabolic adaptations are coordinated by changes in the plasma concentration of key hormones and by tissue-specific variations in hormonal sensitivity and responsiveness (1,2). For example, the secretion of growth hormone (GH)<sup>3</sup> is elevated in early lactation (2,3) and promotes the mobilization of nonesterified fatty acids from adipose tissue and their oxidative use by the rest of the body

(1). However, other GH actions, such as stimulation of hepatic insulin-like growth factor-I (IGF-I) synthesis and plasma IGF-I, are compromised in early lactating dairy cows (4). Molecular events accounting for these discordant changes in GH responsiveness between liver and adipose tissue are poorly understood.

Similarly, primary factors responsible for tissue-specific changes in GH responsiveness have not been fully elucidated in dairy cows. Insulin may be involved in liver because chronic hyperinsulinemia increases plasma IGF-I in mid- and late-lactating dairy cows (5–7), and stimulates hepatic GH receptor (GHR) gene expression (8). The abundance of the GHR protein, however, was not measured in any of these studies. Such measurements are required in the case of the GHR because the relation between mRNA and protein abundance is not simple. This complication arises from the existence of 3 major classes of GHR transcripts [GHR1A, GHR1B, and GHR1C (9)], each translated with a different efficiency (10). In the case of extrahepatic tissues, no information exists on the effects of insulin or physiological state on the abundance of the GHR protein.

To examine the role of insulin, we first characterized the relationship between its plasma profile and those of net energy

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<sup>3</sup> Abbreviations used: CP, crude protein; DM, dry matter; EB, energy balance; EL, early lactation; GH, growth hormone; GHR, growth hormone receptor; IGF-I, insulin-like growth factor-I; IGF-BP, insulin-like growth factor binding protein; LP, late pregnancy; NE<sub>L</sub>, net energy of lactation; PMSF, phenylmethylsulfonyl fluoride; RPA, ribonuclease protection assay; State, physiological state; TMR, total mixed ration.

balance and plasma IGF-I during the periparturient period when the most dynamic changes occur. Second, we performed chronic hyperinsulinemic-euglycemic clamps in late pregnancy when the concentration of plasma IGF-I is normal and in early lactation when it is maximally reduced. Effects of insulin were monitored by measuring indices of IGF-I synthesis and cellular abundance of proteins involved in transmitting the GH signal in liver and adipose tissue (GHR protein, JAK2 and STAT5).

## MATERIALS AND METHODS

**Animals and design.** Two experiments were performed using multiparous (mean parity = 2.7, range = 2–4) Holstein cows. In each case, experimental procedures were conducted with the guidance and approval of the Cornell University Institutional Animal Care Committee.

**Temporal relations between plasma IGF-I, insulin, and energy balance.** Cows ( $n = 9$ ) were studied in the period from d 21 prepartum to d 28 postpartum (d -21 to +28 relative to parturition on d 0). They consumed a total mixed ration (TMR) ad libitum once daily. Net energy of lactation ( $NE_L$ ) and crude protein (CP) content of the TMR was 6.52 MJ and 141 g/kg dry matter (DM) between wk -3 and parturition, and 7.19 MJ and 179 g/kg DM after parturition. Cows were weighed at weekly intervals throughout the experiment and milked daily at 1130 and 2330 h during lactation. Blood samples were obtained thrice weekly between 1000 and 1130 h by coccygeal venipuncture. Plasma was prepared immediately and frozen at  $-20^\circ\text{C}$  until analyzed.

**Effect of insulin on the GH-IGF system.** This study was originally designed to describe the effects of insulin on plasma leptin (11). Briefly, 6 cows were studied in late pregnancy [LP,  $-31 \pm 1.5$  d (mean  $\pm$  SEM)] and again during early lactation (EL,  $+7 \pm 1.6$  d). Cows weighed  $626 \pm 18$  kg in LP and  $582 \pm 15$  kg in EL. They were fed a TMR consisting of 6.52 MJ  $NE_L$  and 140 g CP/kg DM during LP, and 6.64 MJ  $NE_L$  and 198 g CP/kg DM during EL. Feed was consumed ad libitum in 12 bihourly meals, and water was available at all times. After parturition, cows were milked twice daily at 0600 h and 1800 h.

Basal conditions were characterized by taking blood samples over 66 h followed by biopsy of liver and tailhead adipose tissue (11,12). Biopsies were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Immediately after biopsies, bovine pancreatic insulin (lot 615-70N-80, 26.6 IU/mg, Lilly Research Laboratories) was infused i.v. at the rate of 172 nmol/(kg body weight  $\cdot$  h) for 96 h during LP and 48 h during EL. The concentration of blood glucose was determined hourly using a Surestep glucometer (Lifescan) and kept at the level observed during the basal period by varying the rate of glucose (500 g/L dextrose solution, Butler). Additional blood samples were taken during the basal period and every 4 h during insulin infusion. Liver and adipose tissue were biopsied immediately before discontinuing the infusion of insulin.

**Whole-body energetics.** Individual energy intake was estimated from daily feed intake data and energy content of the TMR. During the clamp, the energy contributed by glucose was calculated by multiplying the mass infused by its energy value (15.3 MJ/kg). Maintenance energy requirements were estimated from individual body weight, and milk energy output was calculated from daily milk yield and its energy content [estimated from the fat, lactose, and protein content of milk (13)]. Estimates of net energy balance (EB) were calculated for each cow by the difference between net energy inputs (energy from consumed TMR and infused glucose) and outputs (maintenance and milk energy) (3,11).

**Analysis of gene expression.** Total RNA was extracted by a modification of the guanidinium thiocyanate-phenol-chloroform method (14). Glycogen was removed from hepatic total RNA by affinity chromatography (RNeasy, Qiagen). The concentration of total RNA was determined by absorbance at 260 nm, and its quality was verified by staining formaldehyde agarose gel with Syber Green II (Molecular Probes).

Hepatic expression of the IGF-I and GHR genes was measured by specific ribonuclease protection assays (RPA) as described (15). Par-

tial DNA fragments corresponding to bovine IGF-I [137 and 63 nt from exons 3 and 4, respectively] and GHR1A mRNA [191, 81 and 40 nt from exons 1A, 2 and 3, respectively] were subcloned in the plasmid Lit 29 (New England Biolabs) for the generation of antisense RNA using [ $^{32}\text{P}$ ]UTP and T7 DNA polymerase (Maxiscript, Ambion). Antisense RNA was hybridized with total RNA (10  $\mu\text{g}$ ) using a commercial kit (RPA III, Ambion). Each RPA was performed in the presence of a 10-fold molar excess of a low-specific-activity 18S riboprobe generated from an 18S DNA template (Ambion). The IGF-I RPA yields a single protected fragment of 200 bp. The GHR RPA yields a fully protected fragment of 312 bp corresponding to the GHR1A transcript, and a partially protected fragment of 121 bp corresponding to the sum of the GHR1B and GHR1C transcripts [GHR<sub>other</sub>] (15,16). Signals were quantified by phosphorimaging and normalized to the 18S signal.

**Analysis of metabolites and hormones.** Concentrations of plasma glucose were determined by the glucose oxidase method (11). Plasma IGF-I, insulin and GH were measured by previously described double antibody RIA (5,11). For each RIA, bovine proteins were used for iodination and standards (recombinant IGF-I, lot GTS-3, Monsanto; rbST, lot 12-77-001, Upjohn; purified insulin, lot 615-70N-80, Lilly Research Laboratories). 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. The primary antibody for the insulin RIA was purchased from Linco Research (guinea pig anti-porcine insulin serum, lot 122-845-P). Secondary antibodies used were caprine anti-rabbit IgG for the IGF-I RIA (lot 12515, Biotech Source), 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). Inter- and intra-assay CV for all assays were  $<8$  and 9%, respectively.

**Western immunoblot analysis.** Frozen tissues (500 mg for liver, 1 g for adipose tissue) were homogenized in 2 mL of 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  $\text{Na}_3\text{VO}_4$ , 1 mmol/L Na pyrophosphate, 10 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 mg/L aprotinin, 10 mg/L leupeptin]. Homogenates were clarified by centrifugation ( $10,000 \times g$  for 20 min at  $4^\circ\text{C}$  followed by  $12,000 \times g$  for 10 min at  $4^\circ\text{C}$ ). In addition, a preparation enriched in membrane protein was obtained by homogenizing 1 g of adipose tissue in 5 mL of sucrose buffer (50 mmol/L Tris, pH 7.6, 250 mmol/L sucrose, 5 mmol/L EGTA, 150 mmol/L NaCl, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1 mmol/L Na pyrophosphate, 10 mmol/L NaF, 1 mmol/L PMSF, 10 mg/L aprotinin, 10 mg/L leupeptin). After clarification ( $1000 \times g$  for 5 min at  $4^\circ\text{C}$ ), homogenates were centrifuged ( $100,000 \times g$  for 60 min at  $4^\circ\text{C}$ ). The pellet was rocked overnight at  $4^\circ\text{C}$  in lysis buffer. Unsuspended materials were removed by centrifugation ( $12,000 \times g$  for 10 min at  $4^\circ\text{C}$ ).

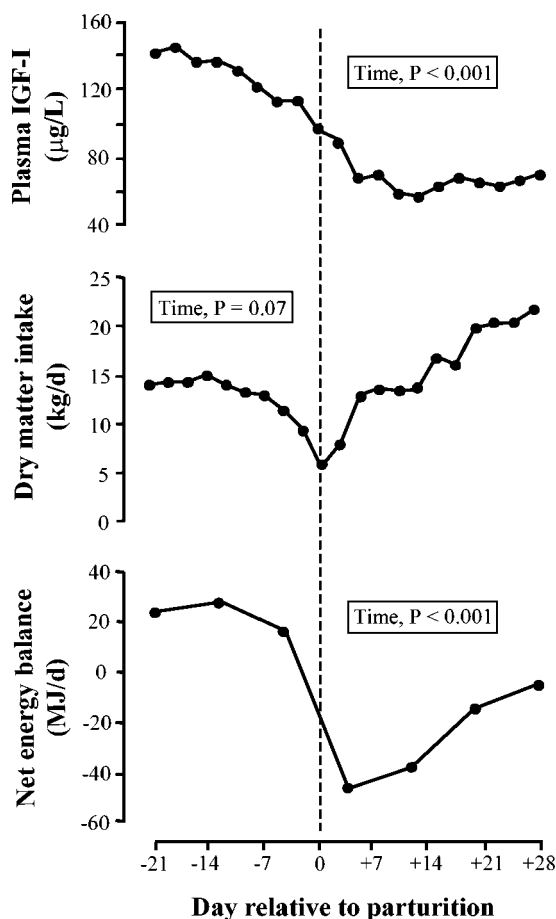
Protein contents of lysates were measured by the bicinchoninic acid protein assay (Pierce). Lysates were electrophoresed on 10% discontinuous SDS-polyacrylamide gels (see figure legends for protein amount) and transferred overnight to nitrocellulose membranes (Schleicher and Schuell). The membranes were immersed for 1 h at room temperature in blocking solution (50 mmol/L Tris, pH 7.4, 200 mmol/L NaCl, 1 mL/L Tween 20, 50 g/L nonfat dried skim milk). Membranes were incubated with primary antibodies against the GHR [rabbit anti-GHR<sub>cyt-AL47</sub> raised against a bacterially expressed N-terminally His-tagged fusion protein incorporating human GHR residues 271-620 (17)], HNF4 (rabbit anti-human HNF4, Geneka Biotechnology), STAT5b (rabbit anti-mouse STAT5b, #sc-835, Santa Cruz Biotechnology), STAT3 (rabbit anti-mouse STAT3, #sc-482, Santa Cruz Biotechnology), Sp1 (rabbit anti-rat Sp1, Santa Cruz Biotechnology) and JAK2 [rabbit anti-JAK2<sub>AL33</sub> directed at residues 746-1129 of murine JAK2 (18)]. These antibodies were validated in bovine extracts and shown to detect proteins of  $\sim 130$  and 140 kDa for the GHR,  $\sim 55$  kDa for HNF4,  $\sim 120$  kDa for JAK2, 92 kDa for STAT3, 95 kDa for STAT5, and  $\sim 95$  and 106 kDa for Sp1 (16,19,20). Primary antibodies were diluted in blocking solution (GHR and HNF4, 1:1000; STAT5, STAT3, Sp1 and JAK2, 1:2000). After incubation, membranes were washed 5 times (5 min each) followed by incubation with the secondary antibody (goat anti-rabbit

IgG-horseradish peroxidase, KPL) at a 1:5000 dilution in blocking solution for 1 h at room temperature. The membranes were washed as above and incubated with LumiGLO Western blot chemiluminescence (KPL). Chemiluminescence was detected by exposure to film. Signals were analyzed by densitometric scanning and NIH Image 1.63 software.

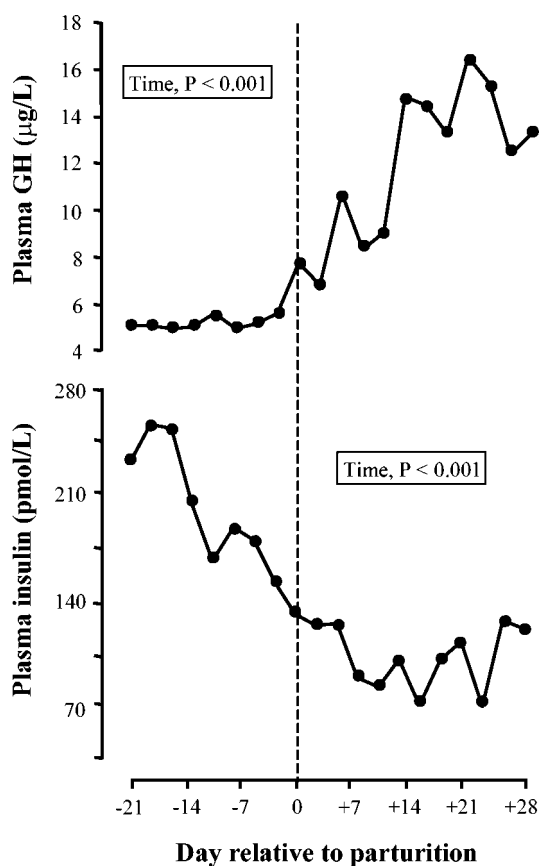
**Statistical analyses.** In the temporal experiment, samples were obtained at fixed times each week. To accommodate natural variation in time of parturition, samples were grouped by a 3-d interval (i.e., day -21 represents samples obtained on d -22, -21, or -20). Data were analyzed by a mixed model accounting for time as the fixed effect and animal as the random effect. For the insulin experiment, means were calculated for the energy-related variables over the last 48 h of the basal period and over the 48 h of the clamp period. Data were analyzed by a general linear model accounting for physiologic state (State, LP vs. EL), insulin (Insulin, basal vs. insulin infusion) and their interaction (State  $\times$  Insulin) as fixed effects and animal as the random effect. Main effects and their interactions were declared significant at  $P < 0.05$  and  $P < 0.10$ , respectively.

## RESULTS

**Relation between plasma IGF-I, insulin, and net EB during the periparturient period.** The plasma concentration of IGF-I was  $\sim 140$   $\mu\text{g/L}$  until a decline was initiated 7–10 d before parturition (Fig. 1,  $P < 0.001$ ). This decline was



**FIGURE 1** Profiles of plasma IGF-I, dry matter intake, and net energy balance during the transition from pregnancy to lactation in multiparous dairy cows. Cows ( $n = 9$ ) were studied in the period from d 21 prepartum to d 28 postpartum (d -21 to +28 relative to parturition on d 0). The day of parturition is denoted by the dashed vertical line. Pooled SE were 8  $\mu\text{g/L}$  for plasma IGF-I, 1.2 kg/d for dry matter intake, and 6.3 MJ/d for net energy balance.



**FIGURE 2** Profiles of plasma GH and insulin during the transition from pregnancy to lactation in multiparous dairy cows. Cows ( $n = 9$ ) were studied in the period from d 21 prepartum to d 28 postpartum (d -21 to +28 relative to parturition on d 0). The day of parturition is denoted by the dashed vertical line. Pooled SE were 3.0  $\mu\text{g/L}$  for GH and 34.4 pmol/L for insulin.

associated temporally with a depression in feed intake and a reduction in net EB (Fig. 1,  $P = 0.07$  and  $P < 0.001$ , respectively). After parturition, the plasma concentration of IGF-I reached a nadir of  $\sim 60$   $\mu\text{g/L}$  on d +10, in conjunction with near maximal milk energy output and net EB deficit (results not shown and Fig. 1). The decline in plasma IGF-I matched almost exactly the profile of plasma insulin between d -7 and +21, and occurred despite a postparturient rise in plasma GH (Fig. 2,  $P < 0.001$ ).

**Insulin increases IGF-I in early lactation.** Next, we asked whether insulin could increase IGF-I in EL when its plasma concentration was maximally depressed on d +10; for comparison, effects of insulin were also measured in LP (starting on d -28), well before the periparturient depression was initiated. As expected, the preclamp concentration of plasma IGF-I was depressed in EL (LP vs. EL, 172 vs. 80  $\mu\text{g/L}$ ,  $P < 0.01$ ), and was associated with negative net EB (LP vs. EL, 37.6 vs. -61.0 MJ/d,  $P < 0.001$ ), reduced plasma insulin and glucose (316 vs. 123 pmol/L and 2.72 vs. 2.27 mmol/L,  $P < 0.001$ ) and increased plasma GH (3.5 vs. 4.6  $\mu\text{g/L}$ ,  $P < 0.05$ ).

The effects of euglycemic hyperinsulinemia on whole-body energetics were reported recently (11). Briefly, insulin infusion increased plasma insulin from 316 to 701 pmol/L during LP and from 123 to 438 pmol/L during EL ( $P < 0.001$ ). In LP, net energy intake did not differ between basal and clamp periods (basal vs. insulin, 86.1 vs. 81.9 MJ/d). During EL, however,

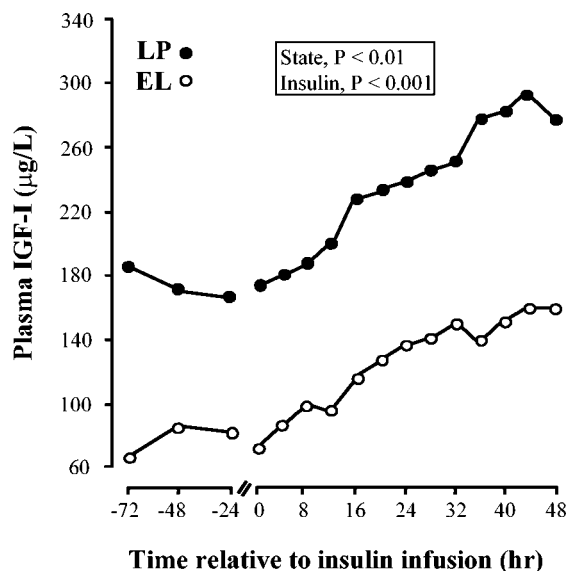
hyperinsulinemia caused a 33% reduction in net energy intake (basal vs. insulin, 115.9 vs 76.1 MJ/d,  $P < 0.01$ ) as well as a 15% reduction in milk energy output (130.9 vs. 113.4 MJ/d,  $P < 0.05$ ). When the energy value of infused glucose is considered, hyperinsulinemia caused similar improvement in net EB during both states (+21.3 and +13.0 MJ/d during LP and EL, respectively). Despite this improvement, the net EB of EL remained substantially negative (-48.1 MJ/d).

During both states, plasma IGF-I rose with similar kinetics to reach near asymptotic values by 48 h (Fig. 3). This was confirmed during LP by comparable plasma IGF-I concentrations after 48 and 96 h of insulin infusion (289 vs. 329  $\mu\text{g/L}$ ,  $P > 0.1$ , results not shown). Irrespective of physiologic state, plasma concentrations of IGF-I during the 36- to 48-h interval of hyperinsulinemia were nearly twice those measured during the basal period (172 vs. 289  $\mu\text{g/L}$  during LP and 80 vs. 159  $\mu\text{g/L}$  during EL,  $P < 0.001$ , Fig. 3). The IGF-I response was associated with increased plasma GH during EL (4.6 vs. 7.3  $\mu\text{g/L}$ ) and with decreased GH during LP (3.5 vs. 2.6  $\mu\text{g/L}$ , State  $\times$  Insulin,  $P = 0.1$ ). Therefore, insulin increases plasma IGF-I independently of physiologic state or energy balance.

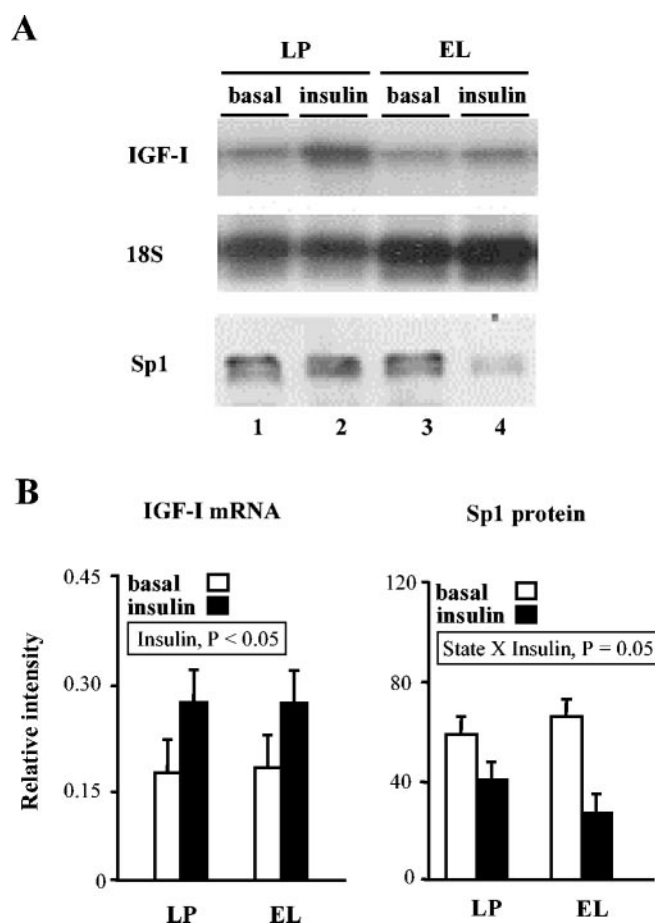
To understand the basis for the effect of insulin, levels of IGF-I mRNA were compared in liver biopsies obtained immediately before the start and termination of the clamp. Insulin infusion increased IGF-I mRNA expression equally well in LP and EL ( $P < 0.05$ , Fig. 4). Sp1, a transcription factor mediating some of the positive effects of insulin on IGF-I in rats (21), was reduced by insulin in bovine liver, particularly during EL (State  $\times$  Insulin,  $P = 0.05$ , Fig. 4). This indicates that, in dairy cows, insulin increases plasma IGF-I by stimulating hepatic production, but these effects do not necessitate greater Sp1 abundance.

#### Effect of insulin on GH signaling components in liver.

Effects of insulin on IGF-I mRNA could be direct or could represent potentiation of the positive effects of GH on IGF-I gene transcription. The abundance of the GHR protein in total cellular extracts was lower in EL than in LP ( $P < 0.05$ ,



**FIGURE 3** Effects of physiologic state and hyperinsulinemic-euglycemia on plasma IGF-I in multiparous dairy cows. Dairy cows ( $n = 6$ ) were studied under basal conditions (-72 to -24 h relative to infusion of insulin) and during a 48-h period of hyperinsulinemic-euglycemia in late pregnancy (LP) and early lactation (EL). The pooled SE was 19.5  $\mu\text{g/L}$ .



**FIGURE 4** Effects of physiologic state and hyperinsulinemic-euglycemia on IGF-I mRNA and Sp1 protein abundance in liver of multiparous dairy cows. Dairy cows ( $n = 6$ ) were studied in late pregnancy (LP) and early lactation (EL). Liver biopsies were obtained under basal conditions (basal, d -28 during LP and d +10 during EL) and at the end of the period of hyperinsulinemic-euglycemia (insulin, d -24 during LP and d +12 during EL). (A) Levels of IGF-I mRNA and Sp1 protein were measured by RPA and Western immunoblotting, respectively. Data are shown for 1 representative cow. (B) Bars represent the means  $\pm$  SEM of the IGF-I signal (normalized to 18S) and Sp1 signal.

Fig. 5), and was increased by insulin irrespective of physiologic state ( $P < 0.05$ ). In contrast, insulin had no effect on the abundance of intracellular proteins involved in GH signaling (JAK2, STAT3 and STAT5, Fig. 5). Tyrosine phosphorylation of these STAT was unaffected by insulin (STAT3) or not detected (STAT5; results not shown). These results suggest that insulin acts as a rheostat of hepatic GHR synthesis.

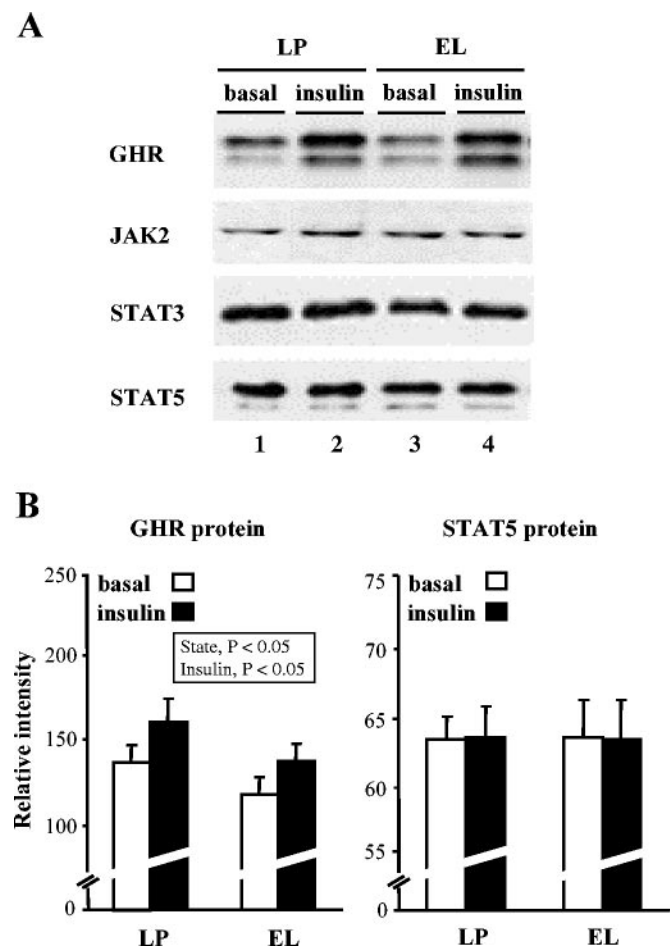
To determine whether insulin increased biosynthesis of the receptor, we measured the abundance of the liver specific GHR transcript (GHR1A) as well as the abundance of all other transcripts (GHR<sub>other</sub>) (Fig. 6). The abundance of the GHR1A transcript tended to increase during insulin infusion ( $P = 0.08$ ), and was higher during LP than during EL ( $P < 0.04$ ). Because GHR1A is produced by an HNF4-dependent promoter (22), we asked whether variation in the abundance of this transcription factor could explain the effects of physiologic state and insulin. However, hepatic abundance of HNF4 did not change between LP and EL when GHR1A abundance decreased, and was even reduced by insulin during EL (Fig. 6, State  $\times$  Insulin,  $P = 0.05$ ) when GHR1A abundance tended to increase. Abundance of GHR<sub>other</sub> was not

affected by physiologic state or insulin. These data suggest that in liver, insulin and physiologic state alter GHR abundance by regulating the levels of the liver-specific GHR1A transcript.

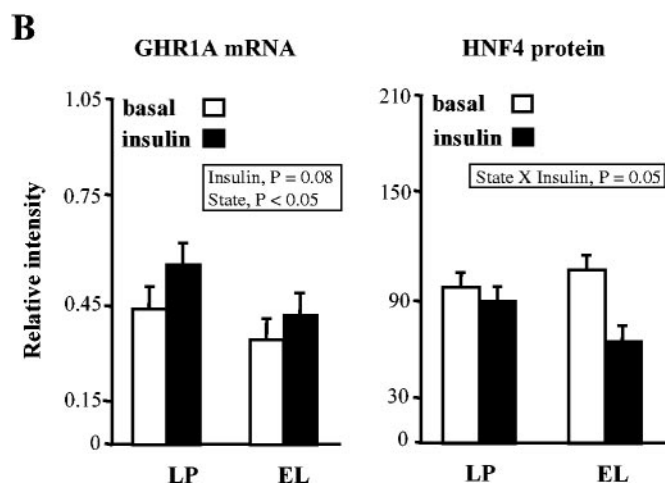
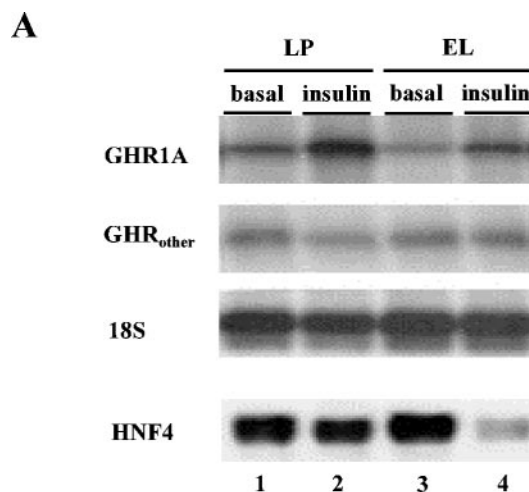
**Effect of insulin on GHR abundance in adipose tissue.** Finally, we asked whether physiologic state and insulin exerted any effects on GHR abundance in adipose tissue. GHR abundance was lower in EL than in LP (Fig. 7,  $P < 0.01$ ), and insulin doubled GHR abundance irrespective of physiologic state ( $P < 0.01$ ). During LP, this positive effect of insulin occurred in the absence of changes in GHR<sub>other</sub> expression (results not shown). Whether GHR<sub>other</sub> expression was similar during LP and EL could not be determined due to the lack of tissue during EL.

## DISCUSSION

The present study shows that the reduction in plasma IGF-I is initiated when feed intake starts to decline in late preg-



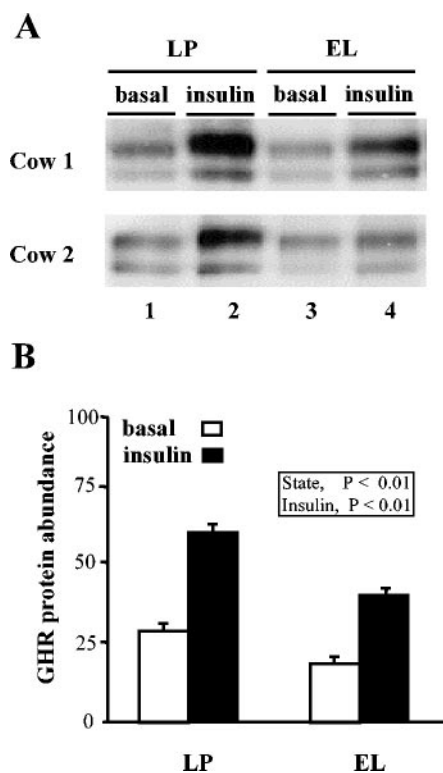
**FIGURE 5** Effects of physiologic state and hyperinsulinemic-euglycemia on the abundance of the GHR, JAK2, and STAT proteins in liver of multiparous dairy cows. Dairy cows ( $n = 6$ ) were studied in late pregnancy (LP) and early lactation (EL). Liver biopsies were obtained under basal conditions (basal, d -28 during LP and d +10 during EL) and at the end of the period of hyperinsulinemic-euglycemia (insulin, d -24 during LP and d +12 during EL). (A) Levels of the GHR, JAK2, STAT3, and STAT5 proteins were measured by Western immunoblotting. Data are shown for 1 representative cow. (B) Bars represent means  $\pm$  SEM of GHR and STAT5 signals. The JAK2 and STAT3 signals were also quantified but were not affected by either physiologic state or insulin.



**FIGURE 6** Effects of physiologic state and hyperinsulinemic-euglycemia on GHR mRNA and HNF4 protein abundance in liver of multiparous dairy cows. Dairy cows ( $n = 6$ ) were studied in late pregnancy (LP) and early lactation (EL). Liver biopsies were obtained under basal conditions (d -28 during LP and d +10 during EL) and at the end of the period of hyperinsulinemic-euglycemia (insulin, d -24 during LP and d +12 during EL). (A) Levels of the GHR1A and GHR<sub>other</sub> transcripts were measured by RPA. Levels of the HNF4 protein were measured by Western immunoblotting. Data are shown for 1 representative cow. (B) Bars represent the means  $\pm$  SEM of the GHR1A signal (normalized to 18S) and HNF4 signal. The GHR<sub>other</sub> signal was also quantified but was not affected by either physiologic state or insulin.

nancy. After parturition, milk secretion becomes the major determinant of energy balance, and plasma IGF-I remains low over the first few weeks of lactation despite increasing feed intake. Overall, these data agree with previous studies suggesting that in dairy cattle, development of a nutritional deficit near parturition contributes to the reduction in plasma IGF-I (3,4).

In the immediate prepartum period, the fall of plasma insulin nearly mirrors that of net EB, suggesting a role in mediating effects of EB on plasma IGF-I. Moreover, insulin increases the plasma concentration of IGF-I in later lactating dairy cows when in equilibrium or positive EB (5-7). We now showed that these positive effects of insulin occur in LP and remain surprisingly potent in EL when cows are in negative EB. Three additional points must be made regarding the plasma IGF-I response. First, the insulin infusion rate we used



**FIGURE 7** Effects of physiologic state and hyperinsulinemic-euglycemia on the GHR in adipose tissue. Dairy cows ( $n = 6$ ) were studied in late pregnancy (LP) and early lactation (EL). Adipose tissue biopsies were obtained under basal conditions (basal, d  $-28$  during LP and d  $+10$  during EL) and at the end of the period of hyperinsulinemic-euglycemia (insulin, d  $-24$  during LP and d  $+12$  during EL). (A) The abundance of the GHR was measured by Western immunoblotting. Data are shown for 2 representative cows. (B) Bars represent means  $\pm$  SEM of GHR signals.

produced greater steady-state plasma insulin and IGF-I concentrations in LP than in EL, but the net increase in plasma IGF-I was identical. In the absence of additional insulin doses, however, we cannot draw conclusions about either the sensitivity or responsiveness of the IGF-I system. Nevertheless, it is remarkable that insulin infusion during EL raised plasma insulin to the basal levels of LP and completely eliminated the depression of plasma concentration of IGF-I. Second, the stimulation of hepatic IGF-I mRNA by insulin in both LP and EL indicates that increased synthesis accounted, at least in part, for the plasma IGF-I response. This was expected given that most of the plasma IGF-I originates in liver (23) and the ability of insulin to increase hepatic IGF-I mRNA and plasma IGF-I in other species during insulin-deficient conditions (21,24). Our design does not resolve whether increased glucose availability is required for insulin to increase plasma IGF-I. However, we believe that insulin is the most important factor because hepatic glucose transport is concentration dependent (25) and presumably constant under euglycemic conditions. Finally, in agreement with previous studies (5,8), insulin also altered the plasma concentration of IGFBP-2 and IGFBP-3 (R. P. Rhoads and Y. R. Boisclair, unpublished results), but whether these changes contributed to the plasma IGF-I response or are only a consequence of increased hepatic IGF-I production is unclear at this time (26,27).

We envision 2 possible models to explain the positive effects of insulin on hepatic IGF-I gene expression in periparturient dairy cattle. First, insulin could increase IGF-I gene

transcription directly, as shown in rat hepatocytes (28,29). In this context, others have speculated that direct effects of insulin on IGF-I mRNA in bovine liver would involve increased abundance of the transcription factor Sp1 (8). This proposal was based on the positive effects of insulin on Sp1 abundance in rat hepatoma cells and diabetic liver (30), and on the presence of an Sp1 site in 1 of the regions conferring insulin responsiveness to the rat IGF-I gene (31,32). However, we showed that insulin does not increase Sp1 abundance, indicating that in bovine liver, its positive effects on IGF-I mRNA do not require changes in Sp1 abundance. Consistent with our findings, the 25-bp region conferring insulin responsiveness to the rat IGF-I is conserved in the bovine IGF-I gene (92% sequence identity), but the Sp1 site it contains is not [rat vs. cattle, GGGCAG vs. TAGCAG, Genbank #AY277405 and (31)].

Our data are also consistent with a second model in which insulin regulates GHR expression in liver, thereby affecting the ability of GH to increase IGF-I gene transcription. This model was suggested by concomitant reductions in GHR and IGF-I expression at parturition (15,16), and would provide a simple mechanism to explain the minimal GH-mediated IGF-I response of hypoinsulinemic, early lactating dairy cows [(4) and D. E. Bauman and Y. R. Boisclair, unpublished results]. Our data showing that insulin increases hepatic abundance of the GHR protein in both LP and EL indicate that this model is possible in cattle, and agree with reports of insulin-dependent synthesis of the GHR in the human liver cell line HuH7 and in the liver of diabetic rats (33,34). Along the same line, we also asked whether insulin altered the abundance of STAT5, the transcription factor mediating the positive effects of GH on IGF-I gene transcription (35,36), and the abundance of JAK2, the tyrosine kinase responsible for STAT activation (37). Our results indicate that the abundance of these intracellular proteins was not limiting the effects of insulin on IGF-I gene expression. Future studies are warranted to determine whether insulin effects on IGF-I gene expression in bovine liver involve both mechanisms (direct effect on the IGF-I gene and indirect via the GHR), and if so, what is their relative importance.

We found that insulin tended to increase hepatic GHR1A abundance. We also observed that GHR1A abundance was increased by insulin during wk 4 of lactation [(7) and R. P. Rhoads, K. L. Ingvarsen and Y. R. Boisclair, unpublished results]; similar results were reported recently (8). Overall, these data suggest that insulin stimulates hepatic GHR synthesis by increasing GHR1A abundance, and fit with other data in cattle showing that changes in GHR1A expression account for a major part of the variation in total GHR mRNA seen in liver with development, nutrition and parturition (15,16,38). The bovine GHR promoter responsible for GHR1A synthesis is active only in liver and is stimulated by the liver-enriched transcription factor HNF4 (22). In LPS-treated rats (39), the reduction in GHR abundance and GH stimulated IGF-I production seen after LPS administration is associated with decreased HNF4 abundance (40). However, we were unable to discern any relation between HNF4 abundance and GHR1A expression in cattle liver. It remains possible, however, that insulin induces post-translational modifications that stimulate the activity of HNF4 (41).

Our RPA assay provided a measure of hepatic expression of the non-GHR1A transcripts, which in cattle liver, represent mainly GHR1B [ $\sim 74\%$  of the non-GHR1A transcripts according to Jiang and Lucy (10)]. Hepatic GHR1B or GHR<sub>other</sub> signals were shown to be invariant under most conditions, including the transition from pregnancy to lactation (15,16)

and hyperinsulinemia (this study). In contrast to our findings, Butler et al. (8) proposed that insulin inhibited the expression of non-GHR1A transcripts based on unchanged total GHR expression in the face of increased GHR1A abundance. This result may reflect a technical problem, such as standardization of GHR expression to that of cyclophilin, a mRNA that is regulated by insulin in the liver of early lactating cattle (42).

A second major target of GH is adipose tissue in which it favors export of lipids by inhibiting insulin-mediated lipogenesis and amplifying the lipolytic response to  $\beta$ -adrenergic signals (43). These actions of GH are thought to be relatively insensitive to changes in the plane of nutrition (1,44). A recent report, based entirely on GHR mRNA data, suggested that GHR was increased in the adipose tissue of early lactating dairy cows, and implicated the hypoinsulinemia of this period as the primary cause (8). Our data clearly show that these conclusions are wrong because the abundance of the GHR protein fell as the plasma insulin concentration decreased from LP to EL, and was increased by exogenous insulin infusion in both states. We were unable to measure GHR gene expression during EL, but during LP, the positive effects of insulin were not associated with an increase in GHR<sub>other</sub>. In adipose tissue of well-fed cattle, the GHR<sub>other</sub> reflects predominantly GHR1B and presumably some GHR1C (45). Insulin could increase GHR abundance by increasing the relative abundance of the more efficiently translated GHR1C at the expense of GHR1B (10), or more simply, by stimulating the translation of the preexisting mRNA population (46). These data also illustrate that given the complexities associated with GHR biosynthesis in every tissue [i.e., multiple transcripts differing in translation efficiency (10)], measurements of the GHR protein are preferable to mRNA data whenever possible.

In summary, we showed that insulin increases hepatic IGF-I synthesis, and that this effect could be mediated in part by increased GHR protein in liver. Positive effects of insulin on the abundance of the GHR were also seen in adipose tissue. Our data show that the periparturient dairy cow is a useful model with which to study the mechanisms by which energy insufficiency and insulin alter GHR abundance, and the roles played by the GHR in regulating tissue-specific changes in GH responsiveness.

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