Effect of insulin and growth hormone on plasma leptin in periparturient dairy cows

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In high-yielding dairy cows, the onset of lactation increases the total energy requirements by approximately fourfold, reflecting mostly the oxidative and milk precursor needs of the mammary gland (5, 6). Because the hyperphagia required to meet these additional demands develops slowly, shortfalls are met by mobilization of endogenous reserves and by shifting the pattern of nutrients used by nonmammary tissues (5, 6). Peripheral mechanisms orchestrating these adaptations have been studied extensively in ruminants, and they involve changes in the concentration and actions of hormones (51). For example, hypoinsulinaemia and decreased insulin responsiveness of skeletal muscle and adipose tissue occur simultaneously in early lactation (7, 51). The sum effect of these adaptations is increased availability of glucose for the mammary gland where uptake is independent of insulin (5, 7).

Some of these adaptations, such as hypoinsulinaemia, also occur in lactating rodents and humans (40, 49).

The central nervous system (CNS) also plays important regulatory roles during this period and is receiving growing attention (45, 47, 49). This renewed interest reflects, in part, the recent discovery of leptin, a protein hormone synthesized almost exclusively by white adipose tissue (AT) (1, 29). In contrast to most other metabolic hormones, leptin acts predominantly on regions of the brain involved in the regulation of energy metabolism, such as the arcuate, ventromedial, and dorsomedial nuclei of the hypothalamus (1, 43, 45).

Ahima and Flier (1) proposed that a fall in plasma leptin signals to the CNS that a state of energy insufficiency prevails in the periphery. Consistent with such a role during the transition period, plasma leptin is reduced around parturition in dairy cows (8, 31, 33). Moreover, early lactation and other conditions in which plasma leptin is low or absent share many adaptations such as depressed reproductive and immune function and higher metabolic efficiency (1, 5, 45, 49).

Factors responsible for the decline of plasma leptin in periparturient dairy cows have not been characterized. Adiposity cannot be the primary cause because plasma leptin falls before significant depletion of lipid reserves occurs (8). The decline, however, matches almost exactly the profile of plasma insulin at that time (6, 8). Insulin stimulates plasma leptin in simple-stomached species such as rodents and humans (1, 12),...
but its role in ruminants remains unknown (29). Moreover, growth hormone (GH), a hormone that attenuates many actions of insulin in bovine AT (19, 51), is also increased at parturition (6, 8). Therefore, the objectives of this study were to determine whether insulin and GH could regulate plasma leptin in periparturient dairy cows. Our studies show that insulin increases plasma leptin to a greater extent during pregnancy than during lactation and that GH is unlikely to play a major role.

MATERIALS AND METHODS

Animals and design. The Cornell University Institutional Animal Care and Use Committee approved all experimental procedures. Only multiparous Holstein dairy cows were used.

Effect of insulin. Six cows were studied in late pregnancy (LP) and in early lactation (EL). At the start of these periods, cows averaged 31 ± 1.5 days prepartum (mean ± SE) and 7 ± 1.6 days postpartum, respectively, and weighed 626 ± 18 and 582 ± 15 kg, respectively. Cows were housed in a controlled environment (20°C, lights on 0600–2100) and fed a total mixed ration (TMR). Cows were fed according to recommended feeding standards (39). The TMR contained 1.56 Mcal of net energy for lactation (NEL) and 140 g crude protein (CP)/kg dry matter (DM) during LP and 1.58 Mcal NEL, and 198 g CP/kg DM during EL. The TMR was offered ad libitum in 12 binhoars with water available at all times. During EL, cows also consumed a daily allowance of long-stem hay (3.6 kg containing 1.23 Mcal NEL, and 144 g CP/kg DM). After parturition, cows were milked twice daily at 0600 and 1800.

Hyperinsulinemic-euglycemic clamps were performed by previously described procedures (22, 35). Briefly, cows were fitted with chronic indwelling catheters in each external jugular vein. Starting the next day (31 days prepartum during LP and 7 days postpartum during EL), basal conditions were characterized by taking blood samples over 66 h, followed by biopsies of subcutaneous AT from the tailhead region (8). Biopsied tissue was snap frozen in liquid nitrogen and stored at −80°C. Immediately after biopsy, insulin was infused at the rate of 1 μg·kg body wt·h−1 for 96 h during LP and for 48 h during EL. Infusates were prepared by diluting bovine pancreatic insulin (lot 615-70N-80, 26.6 IU/ mg; Lilly Research Laboratories, Indianapolis, IN) in sterile saline with individual cow plasma as a carrier (2.5% vol/vol). The concentration of blood glucose was determined at frequent intervals (5–15 min) for the first 2–3 h and hourly thereafter with a Surestep glucometer (Lifescan, Milpitas, CA). Plasma glucose was maintained to the concentration observed during the basal period by varying the rate of intrajugular infusion of a glucose solution (50% wt/vol dextrose solution; Abbott Laboratories, Morgan Hills, CA). Blood samples were taken during the basal period and every 4 h during insulin infusion. After addition of heparin (10 IU/ml), plasma was obtained by centrifugation and stored at −20°C until being analyzed for hormones and metabolites. A second AT biopsy was obtained just before the insulin infusion was discontinued.

Source of mammary tissue and cells. Mammary gland and subcutaneous AT were obtained at death from lactating dairy cows and immediately frozen at −80°C. For isolation of mammary epithelial cells, parenchymal tissue was dissected from the mammary glands of additional EL dairy cows. Mammary epithelial cells were isolated by digestion with a mixture of collagenase, hyaluronidase, and elastase, followed by gradient centrifugation (34). This procedure yields primarily mammary epithelial cells (>95% of isolated cells), as shown by morphology and expression of milk protein genes and epithelial cell markers when cultured in vitro (34). Isolated cells were frozen immediately in liquid nitrogen until total RNA was prepared.

Effect of GH. Twelve cows were used in the period from 5 wk prepartum to 5 wk postpartum (weeks −5 to +5 relative to parturition). They were housed in individual tie stalls and fed a TMR ad libitum once daily. Nutrient composition of the TMR varied according to physiological state, with respective NE, and CP content of 1.36 Mcal and 130 g/kg DM during week −5, 1.56 Mcal and 141 g/kg DM during week −4 and parturition, and 1.72 Mcal and 179 g/kg DM after parturition. Cows were randomly allocated to a control or a GH group. The GH group received a daily injection of recombinant bovine GH bovine somatotropin (bST), 45 mg/injection im; lot 96J-B5128-002, Monsanto, St. Louis, MO) on three consecutive days during weeks −5, −2, +1, and +5. Injections were initiated on the second day of each week, corresponding to days −30 ± 4, −13 ± 2, +2, and +30; variation during pregnancy reflects differences between predicted and actual time of parturition. By this mode of administration, GH-induced changes in insulin secretion and AT occur within 1 day of injection and persist for over 24 h after last administration (26, 52). Blood samples were obtained immediately before GH administration (day 0) and the day after the last injection (day 4). Sampling was by coccygeal venipuncture between 1000 and 1130. Control cows were sampled on the same day of each week. Plasma was prepared immediately and frozen at −20°C until analyzed. Cows were weighed at weekly intervals throughout the experiment and milked twice daily at 1130 and 2330 during lactation.

Whole body energetics. Individual feed intakes were recorded on a daily basis. Feed samples were collected weekly and analyzed for nutrient and chemical composition (Dairy One Cooperative, Ithaca, NY). Milk was weighed and sampled at each milking. Individual milk samples (insulin experiment) or weekly composites (GH experiment) were analyzed for protein, fat, and lactose content by infrared analysis (Dairy One Cooperative). Chemical composition of feeds and milk was used to estimate their energy content according to the National Research Council (NRC) (39). These data and body weights were used to calculate individual estimates of net energy balance (EB) on a daily basis (8, 26). To estimate changes in body fatness, two individuals independently assigned a body condition (BC) score (thin = 1, fat = 5) to each cow at the start and end of each period (8).

Analysis of gene expression. Total RNA was extracted from tissues and mammary epithelial cells by a modification of the guanidium thiocyanate-phenol-chloroform method (8, 41). 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, Eugene, OR). Leptin mRNA was quantified by reverse transcription polymerase chain reaction (RT-PCR) with an RPA III kit (Ambion, Austin, TX) (8). The leptin probe corresponding to nt +64 to +316 (ATG, +1) of the bovine leptin cDNA and contained a sequence derived from the last two exons of the gene. The RPA also included a 10-fold molar excess of a low-specificity 18S probe generated from a DNA template (Ambion). Protected bands (253 bp for leptin, 80 bp for 18S) were resolved on 6% polyacrylamide, 7 M urea gels. Signals were quantified by phosphorimaging with a Fujix-Bio-Image Analyzer BAS 1000 (Fuji Medical Systems, Stamford, CT). When comparing leptin expression between mammary...
and adipose tissues, leptin signals were normalized to the mass of input RNA determined by A260.

Analysis of metabolites and hormones. Plasma glucose was measured by the glucose oxidase method and nonesterified fatty acids (NEFA) by the acyl-CoA synthetase/oxidase method (8). Plasma concentration of β-hydroxybutyrate was assayed with the β-hydroxybutyrate dehydrogenase method (kit 310-UV, Sigma, St. Louis, MO). Plasma concentrations of insulin and GH were determined by established RIAs (8). The concentration of leptin in plasma and milk was measured by a double-antibody bovine RIA developed in our laboratory (16). Milk leptin was assayed exactly as plasma, except that it was extensively mixed and sonicated before being diluted in assay buffer (27). RIA performed adequately with milk samples, as shown by parallel displacement curves of 125I-labeled bovine leptin, by serial dilution of bovine milk and standard, and by quantitative recovery of recombinant bovine leptin added to whole milk (>85%). The RIA for leptin has a sensitivity of 0.5 ng/ml and a range of 0.5–20 ng/ml. Inter- and intra-assay coefficients of variation for all metabolic and hormone assays averaged <8% and 9%, respectively.

Statistical methods. For the insulin study, energy-related variables during LP and EL were averaged over the last 48 h of the basal period and over the first 48 h of the clamp period. For hormones and metabolites, averages were calculated at steady state (entire basal period and the 36–48 h clamp period). Data were analyzed by a general linear model accounting for physiological state (STATE, LP vs. EL), insulin (INS, basal vs. hyperinsulinemia), and their interaction (STATE × INS) as fixed effects and animal as the random effect. Linear regression was used to assess the relationship of leptin concentration between plasma and milk. A repeated-measure model was used to analyze the effect of time and insulin on milk leptin concentration and output. For the GH study, the response was calculated for each variable as the difference between day 4 and day 0. Responses were analyzed by a model accounting for GH (GH, control vs. GH), time (TIME, weeks −5, −2, +1, and +5), and their interaction (GH × TIME) as fixed effects and animal as the random effect.

RESULTS

Insulin is a positive regulator of plasma leptin. Basal concentrations of plasma insulin and glucose were 60% and 20% lower in EL than in LP, respectively (Table 1; P < 0.001). Insulin infusion produced an almost immediate increase in plasma insulin concentration of ~2 ng/ml, irrespective of physiological state (Fig. 1 and Table 1; P < 0.001). When expressed relative to basal concentration, steady-state plasma insulin doubled during LP and nearly quadrupled during EL. During the clamps, plasma glucose concentration remained within 10% of basal concentration for both physiological states (Fig. 1), although slightly lower than basal state during the 36–48 h period of both clamps (Table 1; INS, P < 0.05). Despite lower plasma concentrations of glucose and insulin during EL, maintenance of euglycemia required significantly higher rates of glucose infusion (Fig. 1 and Table 2; P < 0.001). These data indicate that clamp conditions were rapidly achieved and maintained during both physiological states (Fig. 1).

Under basal conditions, EL cows consumed 42% more dry matter (Table 2; P < 0.05) and tended to consume 33% more energy than LP cows (P = 0.07). This increase was still insufficient to meet the increased energy expenditures associated with milk production, as shown by the negative net EB during EL (P < 0.001). During EL, cows had lower BC scores and higher plasma NEFA concentrations (Tables 1 and 2; P < 0.05), indicating an increased use of body fat reserves. Despite this accelerated lipid mobilization, there was no evidence of subclinical or clinical ketogenesis, because plasma concentrations of β-hydroxybutyrate did not differ between states (Table 1).

Hyperinsulinemia had no effect on voluntary feed and energy intake during LP but caused a 33% reduction in both variables during EL (Table 2; STATE × INS, P < 0.05). Despite this negative effect, the energy deficit of EL was not exacerbated (STATE × INS, P > 0.15). This is because the decreased feed intake during EL was also associated with a 15% reduction in milk energy output (INS, P < 0.05), reflecting combined reductions in yield as well as in fat and protein content of milk. The negative EB during the EL clamp per-

Table 1. Response in plasma concentrations of hormones and metabolites to hyperinsulinemic-euglycemic clamp during late pregnancy and early lactation

<table>
<thead>
<tr>
<th>Plasma Variable</th>
<th>Late Pregnancy</th>
<th>Early Lactation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Insulin</td>
<td>Basal</td>
</tr>
<tr>
<td>Hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.8</td>
<td>4.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>3.2</td>
<td>5.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Growth hormone, ng/ml</td>
<td>3.5</td>
<td>2.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>49</td>
<td>45</td>
<td>39</td>
</tr>
<tr>
<td>NEFA, μM</td>
<td>95</td>
<td>62</td>
<td>533</td>
</tr>
<tr>
<td>β-Hydroxybutyrate, mg/dl</td>
<td>7.7</td>
<td>5.1</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Multiparous cows (n = 6) were studied during a 66-h basal period (basal) and a 48-h hyperinsulinemic-euglycemic clamp (insulin) in late pregnancy (commencing at 31 ± 1.5 days prepartum) and again in early lactation (7 ± 1.6 days postpartum). Data represent the average of the entire 66-h basal period and the average of the 36–48 h clamp period. Significance level (P) for the effect of physiological state (STATE, late pregnancy vs. early lactation), insulin (INS, basal vs. insulin), and their interaction (STATE × INS) is shown; NS = nonsignificant (P > 0.05). NEFA = nonesterified fatty acids.

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sisted even after estimates were corrected for the caloric value of infused glucose (Table 2).

During both physiological states, plasma leptin started to rise within 4–8 h of insulin infusion and was maximally elevated after 24 h (Fig. 2); no further changes were detected over the next 24 h of hyperinsulinemia during EL or the next 72 h during LP (Fig. 2 and results not shown). During the 36–48 h interval of insulin infusion, plasma leptin was increased nearly 75% during LP but only 13% during EL (Table 1; STATE/H11003/INS, P/H11021/0.05). This modest effect of insulin

**Fig. 1.** Profiles of glucose-related variables during hyperinsulinemic-euglycemic clamps. Six dairy cows in late pregnancy (31 ± 1.5 days prepartum) and early lactation (7 ± 1.6 days postpartum) were studied for 66 h under basal conditions and for 48 h during hyperinsulinemia-euglycemia. Dashed lines on blood glucose graph represent ±10% of baseline blood glucose. Pooled standard errors were 0.1 ng/ml for insulin, 1 mg/dl for plasma glucose, and 1.2 g/h for glucose infusion rate.

**Fig. 2.** Profiles of leptin and nonesterified fatty acids (NEFA) during hyperinsulinemic-euglycemic clamps. Six dairy cows in late pregnancy (31 ± 1.5 days prepartum) and early lactation (7 ± 1.6 days postpartum) were studied for 66 h under basal condition and for 48 h during hyperinsulinemia-euglycemia. Pooled standard errors were 0.1 ng/ml for leptin and 8/M for NEFA.

**Table 2.** Response in energy-related variables to hyperinsulinemic-euglycemic clamp during late pregnancy and early lactation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Late Pregnancy</th>
<th>Early Lactation</th>
<th>SE</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter, kg/day</td>
<td>13.2</td>
<td>12.5</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Energy, Mcal/day</td>
<td>20.7</td>
<td>19.6</td>
<td>1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield, kg/day</td>
<td>37.5</td>
<td>34.6</td>
<td>1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Energy, Mcal/day</td>
<td>31.3</td>
<td>27.1</td>
<td>1.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose infused, g/h</td>
<td>73.4</td>
<td>27.1</td>
<td>4.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Energy balance, Mcal/day</td>
<td>9.0</td>
<td>7.6</td>
<td>2.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Corrected energy balance, Mcal/day</td>
<td>9.0</td>
<td>14.1</td>
<td>1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Body condition score</td>
<td>3.4</td>
<td>3.1</td>
<td>0.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Multiparous cows (n = 6) were studied during a 66-h basal period (basal) and a 48-h hyperinsulinemic-euglycemic clamp (insulin) in late pregnancy (commencing at 31 ± 1.5 days prepartum) and again in early lactation (7 ± 1.6 days postpartum). Data represent the average of the entire 66-h basal period and the average of the 36–48 h clamp period. Significance level for the effect of physiological state (STATE, late pregnancy vs. early lactation), insulin (INS, basal vs. insulin), and their interaction (STATE × INS); NS = nonsignificant (P > 0.05). Glucose infused, average amount of glucose infused during the 36 h to 48 h clamp period. Net energy balance excluding (energy balance) or including (corrected energy balance) the caloric value of infused glucose is shown. Body condition scores were estimated at the start of the basal period during late pregnancy and early lactation.

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on plasma leptin during EL was in contrast to its equally effective inhibition of plasma β-hydroxybutyrate during both states (P < 0.01) and to its exaggerated inhibition of plasma NEFA during EL (Table 1 and Fig. 2; STATE × INS, P < 0.05). We conclude that insulin is capable of increasing plasma leptin in dairy cows but this effect is attenuated considerably during EL.

To examine the mechanism underlying the effects of insulin on plasma leptin, AT was obtained immediately at the end of the basal and clamp periods and assayed for abundance of leptin mRNA. Hyperinsulinemia increased the abundance of leptin mRNA during LP (Fig. 3; P < 0.05) but had no detectable effect during EL (results not shown).

Secretion of leptin by bovine mammary gland is not increased by hyperinsulinemia in EL. In humans, the concentration of leptin is higher in milk than in plasma (27, 46). Moreover, epithelial cells derived from human breast tissue express the leptin gene and contain immunoreactive leptin (46). To determine whether the mammary gland is a site of synthesis in cattle, mammary tissue and isolated mammary epithelial cells were assayed for the presence of leptin mRNA. As shown in Fig. 4, leptin gene expression was readily detected in epithelial cells isolated from lactating mammary glands. A longer exposure was needed for detection of the leptin signal in mammary tissue, possibly reflecting the difficulty of obtaining representative samples of the whole gland in large animals. We conclude that, in the mammary gland of lactating dairy cows, epithelial cells are the major site of leptin synthesis. Leptin mRNA abundance in mammary epithelial cells, however, is considerably lower than that of AT (Fig. 4).

We also determined milk leptin content by RIA. On average, the concentration of leptin was approximately three times higher in milk than in plasma (Fig. 5). Plasma and milk leptin were not correlated during either the basal or the clamp periods. The milk leptin concentration appeared to increase over time during the clamp (INS × TIME, P < 0.08), but milk leptin

Fig. 3. Effects of insulin on adipose tissue expression of the leptin gene during pregnancy. Six dairy cows in late pregnancy (31 ± 1.5 days prepartum) were studied for 66 h under basal condition and for 96 h during hyperinsulinemia-euglycemia. Biopsies of adipose tissue were obtained at the end of the periods of basal measurement (–) and euglycemic hyperinsulinemia (+) when the plasma concentration of leptin averaged 3.2 and 5.3 ng/ml, respectively. Total RNA (2 μg) was analyzed simultaneously by a ribonuclease protection assay for the abundance of leptin and 18S ribosomal RNA. Bars represent the means ± SE of leptin signal normalized to the 18S signal. Bars with different letters differ at P < 0.05.

Fig. 4. Expression of the leptin gene in the mammary gland during lactation. Tissues were obtained at death from 2 (adipose tissue) or 3 (mammary gland) lactating dairy cows. Epithelial cells were obtained from 2 independent isolations by digestion of the parenchymal mammary compartment of 2 dairy cows. A: total RNA (either 3 or 30 μg as indicated) was analyzed by ribonuclease assay for the abundance of leptin mRNA. The arrow indicates the position of the single leptin signal of 253 bp (top). Before the ribonuclease assay was performed, 2 μg of each sample was evaluated for quality by denaturing agarose gel electrophoresis and Syber Green II staining (bottom). Lanes 1–6 are from a single autoradiogram exposed for 18 h. B: prolonged exposure (72 h) of autoradiogram corresponding to mammary tissue and epithelial cells (lanes 1–4 from A). The arrow indicates the position of the leptin signal. C: leptin signals obtained in A were quantified by phosphorimaging and corrected for the mass of RNA analyzed. Signals were expressed relative to the mean signal obtained in subcutaneous adipose tissue. Means ± SE with different letters differ at P < 0.05.
output during the basal and clamp periods were identical (Fig. 5). We conclude that in EL the bovine mammary gland synthesizes leptin but that mammary secretion is not stimulated by insulin.

**GH has no effect on plasma leptin in EL and LP.** The reduction of plasma leptin observed during the transition from LP to EL is also associated with increased plasma GH (Table 1, \( P < 0.05; \) Ref. 8). We examined the possibility that GH might decrease plasma leptin in cows at 5 and 2 wk prepartum and again at 1 and 5 wk postpartum. At each time, cows receiving GH were sampled immediately before (day 0) and the day after a 3-day period of GH treatment (day 4). Control cows were sampled at identical times but were not treated.

The transition from pregnancy to lactation caused expected and identical changes in both groups of cows (i.e., onset of negative net EB and decreased plasma concentrations of insulin, glucose, and leptin; Fig. 6 and results not shown). As observed by others (52), GH treatment caused a significant increase in plasma insulin during pregnancy but not during lactation (Fig. 6; \( \text{GH} \times \text{TIME}, \ P < 0.05\)). In contrast, GH had no effect on plasma leptin during either pregnancy or lactation. Therefore, the periparturient increase in plasma GH is unlikely to contribute to the postpartum reduction in plasma leptin.

**DISCUSSION**

In dairy cattle, the transition from pregnancy to lactation is associated with a reduction in plasma leptin (8, 31, 33). In other species, placental synthesis of leptin (primates) or a soluble form of the leptin receptor (mice) contributes to elevated plasma leptin during pregnancy (21, 25). However, the ruminant placenta, including that of cattle, does not synthesize leptin (15). Moreover, leptin binding activity is negligible when plasma from pregnant cows is incubated with \(^{125}\)I-leptin and assayed by native PAGE or by gel filtration chromatography (R. A. Ehrhardt and Y. R. Boisclair, unpublished results). Instead, we have attributed the periparturient decline in plasma leptin to inhibition of AT leptin synthesis associated with the negative EB of EL (8). This reasoning is supported by three lines of evidence. First, the periparturient decline in plasma leptin is correlated with the onset of negative EB (8, 33). Second, AT leptin gene expression is reduced in EL (8, 47). Finally, when not milked after parturition, dairy cows remain in positive EB and their plasma leptin concentration is identical to that of dairy cows in LP (8).

To understand the regulation of leptin in periparturient dairy cows, we focused on insulin. Insulin stimulates leptin synthesis in rodent and human AT (1, 12) and, unlike other positive regulatory hormones (e.g., prolactin and glucocorticoids), its plasma concentration is decreased markedly in early lactating dairy cows (6, 8). In support of a role for insulin, we show that physiological elevations in plasma concentration increased circulating leptin in both late pregnant and early lactating cows. In both states, plasma leptin responses to insulin took nearly 24 h to plateau. Similar sluggish responses have been observed in humans when euglycemic clamps were performed at modest hyperinsulinemia (42). The slow kinetics of this response may explain why others failed to detect positive effects of insulin in ruminants after acute or short-term elevations in plasma insulin (20, 32). Despite this slow response, we believe the effects of insulin to be direct because insulin alone stimulates indexes of leptin synthesis in ruminant AT explants (28) and because in vivo leptin responses are accelerated considerably when clamps are performed at supraphysiologically concentrations of insulin (4, 48).

![Fig. 5. Mammary gland secretion of leptin during lactation. Six dairy cows in early lactation (7 ± 1.6 days postpartum) were studied for 66 h under basal conditions and for 48 h during hyperinsulinemic-euglycemic clamp. Cows were milked twice daily at 0600 and 1800. Aliquots of milk obtained at each milking (every 12 h) during basal and clamp periods were analyzed for the concentration of milk leptin by RIA. Left: concentrations of leptin in milk secreted over the last 24 h of the basal and clamp periods plotted against the mean plasma concentration of leptin during the corresponding time interval. Right: milk leptin concentration and yield during the 48-h period preceding (basal) and following the initiation of the hyperinsulinemic-euglycemic clamp (Clamp). The only effect approaching significance was the interaction between treatment and time (INS \times \text{TIME}). Pooled SE for milk leptin concentration and yield were 0.4 ng/ml and 11.2 µg/12 h, respectively.](Image)
At steady state, the plasma leptin response to insulin was six times greater in LP than in EL (2.4 vs. 0.4 ng/ml). Insulin infusion produced similar absolute increases in plasma insulin in LP and EL cows, indicating a similar half-life in both states. However, the degree of hyperinsulinemia during lactation was slightly lower, reflecting lower endogenous insulin secretion. This factor alone is unlikely to account for the muted leptin response during EL. First, an eightfold increase in plasma insulin in EL cows produced only a marginally larger response (0.6 ng/ml; W. R. Butler and Y. R. Boisclair, unpublished results). Similarly, small leptin responses were observed in two 96-h clamps performed in later lactating dairy cows (0.4 and 0.8 ng/ml) even though their hyperinsulinemia exceeded that achieved in LP in the present study (6.8 and 11.5 vs. 4.0 ng/ml; Ref. 9). Second, insulin-dependent glucose uptake is impaired in early lactating ruminants (7, 50), and recent evidence in other species indicates that glucose uptake is a primary determinant of AT leptin synthesis (36, 38). In humans, the plasma leptin response during hypoglycemic clamp experiments is more closely related to glucose infusion rates than either glucose or insulin concentration (53). Glucose exerts these effects by increasing the production of metabolites such as UDP-N-acetylg glucosamine, which sense the energy status of the adipocyte, and stimulates leptin synthesis accordingly (12, 13, 54). Whether the reduced plasma leptin response in EL relates to decreased AT glucose uptake and production of UDP-N-acetylg glucosamine remains to be demonstrated in ruminants.

GH inhibits insulin-mediated glucose uptake by AT (19), and the impaired insulin stimulation of glucose disposal in EL coincides with increased GH secretion (7, 8, 51). Moreover, elevated plasma leptin levels seen in GH-deficient humans are corrected by GH therapy, even before a reduction in adiposity occurs (2, 17). These observations prompted us to ask whether GH is a negative regulator of plasma leptin in dairy cows during the periparturient period. We found that exogenous GH treatment did not reduce plasma leptin, either in EL when the plasma concentration of insulin was unaltered or in LP when its concentration was increased by one- to twofold. These results suggest that GH has no independent effect but inhibits insulin-mediated leptin synthesis, an interpretation supported by identical observations in bovine AT explants (28).

Our results in LP, however, are in contrast to those obtained in GH-treated castrate male cattle, which experience simultaneous increases in plasma insulin and AT leptin gene expression (28). The reason for this discrepancy is unclear, but it could relate to the pregnant state of our nonlactating cows. Finally, β-adrenergic signaling inhibits both basal and insulin-mediated leptin secretion in rodent adipocytes (11, 23). In EL, ruminant AT displays increased β-adrenergic responsiveness (6, 51), suggesting that this system could also contribute to the reduction of plasma leptin after parturition.

As reported for other species, we found significant concentrations of immunoreactive leptin in bovine milk (3, 18, 27, 46). Consistent with the possibility that most of the leptin in milk is synthesized by the mammary gland, milk leptin concentration is substantially greater than, and not correlated with, the plasma leptin concentration. Bonnet et al. (10) recently showed that, in sheep, mammary leptin gene expression is highest during the first 80 days of gestation, before
LEPTIN REGULATION BY INSULIN IN PERIPARTURIENT DAIRY COWS

decreasing during the remainder of gestation and during EL. By immunohistochemistry, they mapped leptin expression to differentiating epithelial cells during late gestation and to myoepithelial cells during lactation, a surprising finding given the high leptin content of milk. In contrast, we show that leptin gene expression is significant in mammary epithelial cells, suggesting that they synthesize a significant fraction of milk leptin in lactating dairy cows. In agreement with our findings, mammary epithelial cells from mice and humans have been shown to synthesize leptin (3, 46).

By immunohistochemistry, they mapped leptin with surprising results. These data indicate that, if they exist, the positive effects of both insulin and IGF-I on mammary leptin synthesis must be small.

Exogenous leptin administration reduces voluntary feed intake in most animals, and this effect usually requires 2–4 days of continuous treatment in ruminants (24, 37). Chronic hyperinsulinemic-euglycemic clamps often reduce feed intake in lactating dairy cows (22, 35), but our data do not support a role for leptin in this effect. In the present study, feed intake was unaffected in LP, when the insulin-dependent response in plasma leptin was the highest, and depressed in EL, when the leptin response was small. We have noted a similar lack of association between the depression in feed intake and increased plasma leptin in other clamp studies performed in early and in later lactation [Ref. 9 and Y. R. Boisclair, unpublished data]. This lack of association is also inconsistent with the idea that a decrease in plasma leptin around parturition drives the hyperphagia of EL. This notion has been experimentally dispelled in rodents by showing that feed intake varies in proportion to litter size but independently of serum leptin concentration (14, 49). Moreover, the transition from pregnancy to lactation is not associated with increased leptin resistance in rodents (30, 49). As we (8) and others (49) have suggested, the hypooleptinemia of EL may serve to increase energy conservation by suppressing functions that are dispensable in the short term (e.g., reproduction and immunity) and by promoting increased metabolic efficiency. The lactating dairy cow, with its substantial energy deficit of EL, is an ideal model to determine the role that leptin plays in these adaptations.

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DISCLOSURES

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REFERENCES

LEPTIN REGULATION BY INSULIN IN PERIPARTURIENT DAIRY COWS


