

Increased plasma leptin attenuates adaptive metabolism in early lactating dairy cows

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Abstract

Mammals meet the increased nutritional demands of lactation through a combination of increased feed intake and a collection of adaptations known as adaptive metabolism (e.g., glucose sparing via insulin resistance, mobilization of endogenous reserves, and increased metabolic efficiency via reduced thyroid hormones). In the modern dairy cow, adaptive metabolism predominates over increased feed intake at the onset of lactation and develops concurrently with a reduction in plasma leptin. To address the role of leptin in the adaptive metabolism of early lactation, we asked which adaptations could be countered by a constant 96-h intravenous infusion of human leptin (hLeptin) starting on day 8 of lactation. Compared to saline infusion (Control), hLeptin did not alter energy intake or milk energy output but caused a modest increase in body weight loss. hLeptin reduced plasma glucose by 9% and hepatic glycogen content by 73%, and these effects were associated with a 17% increase in glucose disposal during an insulin tolerance test. hLeptin attenuated the accumulation of triglyceride in the liver by 28% in the absence of effects on plasma levels of the anti-lipolytic hormone insulin or plasma levels of free fatty acids, a marker of lipid mobilization from adipose tissue. Finally, hLeptin increased the plasma concentrations of T_4 and T_3 by nearly 50% without affecting other neurally regulated hormones (i.e., cortisol and luteinizing hormone (LH)). Overall these data implicate the periparturient reduction in plasma leptin as one of the signals promoting conservation of glucose and energy at the onset of lactation in the energy-deficient dairy cow.

Key Words

- ▶ glucose metabolism
- ▶ lipid metabolism
- ▶ liver
- ▶ thyroid hormone

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Introduction

In most mammals, the nutrient demands of lactation are met by a combination of increased voluntary feed intake and adaptive metabolism (Vernon *et al.* 2002).

Adaptive metabolism refers to processes preserving metabolic homeostasis during periods of energy insufficiency and includes mobilization of endogenous

reserves, tissue-specific insulin resistance, and improved metabolic efficiency (Bell 1995, Vernon *et al.* 2002, Boisclair *et al.* 2006). The relative importance of increased feed intake vs adaptive metabolism in support of lactation varies across species. This can be illustrated by comparing the mouse and the modern dairy cow, two species with similar milk energy output when expressed on a metabolic body weight basis (Prentice & Prentice 1988). After parturition, the lactating mouse meets a gradual increase in mammary nutrient demand nearly entirely by a matching increase in feed intake (Johnson *et al.* 2001, Vernon *et al.* 2002). By contrast, the modern dairy cow reaches near peak milk production within days of parturition in the absence of a compensating increase in voluntary appetite (Bell 1995, Block *et al.* 2001, Reist *et al.* 2003). The energy deficit that follows can reach 30% of total energy requirements over the first month of lactation, necessitating sustained reliance on adaptive metabolism (Bell 1995, Vernon *et al.* 2002). Accordingly, the modern dairy cow provides an opportunity to understand factors triggering adaptive metabolism in the context of lactation.

Adaptive metabolism is hormonally regulated as illustrated by adaptations triggered in early lactating dairy cows by interactions among insulin, growth hormone (GH), and insulin-like growth factor (IGF)1. Specifically, the hypoinsulinemia of early lactation causes loss of the liver GH receptor and reduced hepatic IGF1 production (Rhoads *et al.* 2004, Wook Kim *et al.* 2004). As a consequence of the reduced IGF1 feedback, plasma GH increases, promoting insulin resistance and partitioning of available glucose to the mammary gland in which uptake is insulin independent (Bell 1995, Boisclair *et al.* 2006). More recently, we and others have reported that plasma leptin drops by 30–50% during the days surrounding parturition (Block *et al.* 2001, Reist *et al.* 2003, Janovick *et al.* 2011). This rapid reduction in plasma leptin was caused by the onset of negative energy balance rather than depletion of adipose tissue or loss of the placenta (Block *et al.* 2001, Boisclair *et al.* 2006). A similar reduction in plasma leptin in other physiological contexts promotes adaptive metabolism (Rosenbaum & Leibel 2014, Park & Ahima 2015), but whether it plays this role in the energy-deficient, early lactating dairy cow remains unknown. To answer this question, we reversed the leptin deficit of early lactating by chronic intravenous infusion. Leptin infusion did not impact voluntary energy intake or milk production but attenuated adaptations responsible for glucose and energy conservation in early lactation.

Materials and methods

Animals and design

All experimental procedures were performed at Cornell University (Ithaca, NY, USA) and approved by the Institutional Animal Care and Use Committee. Two experiments were performed sequentially with ten mature Holstein dairy cows (parity ≥ 2). The first experiment described metabolic changes occurring during the transition from late pregnancy (LP) to early lactation (EL) by studying cows with a common set of procedures between days -32 ± 2.0 and -29 ± 2.0 and between days 6 and 8 (relative to parturition on day 0). Cows were weighed and then fitted with bilateral jugular vein catheters on days -32 and 6 , administered an insulin tolerance test on days -31 and 7 , and sampled frequently on days -29 and 8 (every 10 min for 8 h). The frequent blood samples served for the analysis of plasma variables on an hourly basis (glucose, free fatty acids, leptin, insulin, IGF1, T_3 , and T_4) or every 10 min (GH and luteinizing hormone (LH)). Finally, biopsies of the liver were obtained following frequent blood sampling. Cows were fed total mixed rations formulated for each physiological stage (i.e., 1.5 Mcal net energy of lactation (NE_L) and 140 g of crude protein (CP) per kg dry matter (DM) in LP and 1.5 Mcal NE_L and 180 g of CP per kg DM in EL). Rations were offered in unlimited amounts, once daily in LP and as meals served every 4 h in EL. After parturition, cows were milked twice daily at 6:00 and 18:00 h.

The second experiment assessed the effect of leptin administration on the metabolic adaptations of EL. Starting after the 18:00 h milking on day 8 of EL, cows received an intravenous infusion of either saline solution (Control) or recombinant human leptin (hLeptin, Eli Lilly) for 96 h. hLeptin was dissolved in saline solution with individual cow plasma as a carrier (2.5% vol/vol) and infused at the rate of 61 $\mu\text{g}/\text{kg}$ body weight/day. Both saline and hLeptin solutions were delivered as a 6.25 mL/h infusion using a syringe pump. Frequent blood samples were obtained for the first 6 h and every 4 h thereafter. Other experimental procedures included an insulin tolerance test on day 11, frequent blood sampling on day 12 (every 10 min for 8 h), and biopsies of the liver before discontinuing infusions at 18:00 h on day 12. Cows were fed and milked exactly as described in the EL phase of the first experiment.

For both experiments, individual net energy balance was calculated as the difference between energy intake and energy expenditure (maintenance and pregnancy in LP and maintenance and milk energy in EL) exactly as described by Block and coworkers (Block *et al.* 2001).

Predictive equations were used to estimate the energy content of feed and milk from chemical composition and the maintenance energy requirement from body weight (National Research Council 2001).

Analysis of metabolites and hormones

Methods previously validated in our laboratory were used to analyze triglyceride and glycogen content in the liver and plasma glucose and free fatty acids (Leury *et al.* 2003, Rhoads *et al.* 2004). The insulin tolerance test was performed by intravenous administration of an insulin bolus (1.0 µg/kg body weight bovine insulin, Sigma). Sampling frequency and calculation of the glucose response area were performed exactly as described by Sechen *et al.* (1990). Inter- and intra-assay coefficients of variation for all metabolic assays were <7 and <5%, respectively.

Plasma leptin was measured with double-antibody RIAs capable of detecting only bovine (Ehrhardt *et al.* 2000) or hLeptin (Linco Research, Inc.). The fractional rate of hLeptin elimination in the vascular compartment was estimated with the relationship $C=b(1-e^{-kt})$, where C is the plasma concentration of hLeptin at time t , b is the hLeptin concentration at plateau (average plasma concentration between 24 and 96 h of infusion), and k is the rate constant for elimination (Shipley & Clark 1972). The half-life of hLeptin was then calculated with the relation: half-life= $\ln 2/k$.

Solid-phase RIAs were used to measure total T_3 and T_4 (Diasorin, Stillwater, MN, USA) and cortisol (Siemens). Plasma insulin, IGF1, GH, and LH were measured using double-antibody RIAs previously validated in cattle (Leury *et al.* 2003, Rhoads *et al.* 2004, Butler *et al.* 2006). Analysis of the pattern of GH and LH secretion was performed on samples collected every 10 min on days 8 and 12 of lactation. Parameters analyzed were mean plasma concentration, pulse number, and pulse amplitude. Pulses were identified as described by Karsch *et al.* (1987) and corresponded to increases greater than the assay sensitivity, exceeding the previous value by >3 times the standard deviation, and followed by steady decline consistent with the half-life. Inter- and intra-assay coefficients of variation for all hormone assays were <6 and <8%, respectively.

In vitro bioactivity of hLeptin

hLeptin activity was measured using a bioassay consisting of human embryonic kidney 293 (HEK293) cells transfected with plasmid bOBR_b expressing the full-length

bovine leptin receptor and luciferase plasmid p4xM67-TK-Luc containing four tandem repeats of the STAT3-binding site in front of the minimal thymidine kinase promoter (Besser *et al.* 1999). Construction of plasmid bOBR_b involved reverse transcription of total RNA from bovine hypothalamus followed by high fidelity amplification of the leptin receptor cDNA using procedures validated in our laboratory (Boisclair *et al.* 1996). The resulting cDNA was subcloned into the expression plasmid pcDNA4/myc-His and shown by sequencing to encode a protein with an amino acid sequence identical to the full-length bLeptin receptor (RefSeq NM_001012285.2).

Transfection and luciferase assays were performed as previously described (Boisclair *et al.* 1993). In brief, HEK293 cells were grown to 50% confluence in six well plates in minimal Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum. Each well was transfected overnight with 2.5 µg each of bOBR_b and p4xM67-TK-Luc using the calcium phosphate precipitation. After a 24 h recovery in serum free media, cells were stimulated with either hLeptin, human leptin obtained from the National Hormone and Peptide Program (hLeptin-N; Torrance, CA, USA) or bovine Leptin (bLeptin) (Protein Laboratories, Rehovot, Israel). Luciferase activity was assayed 6 h later using the Dual Luciferase reporter system (Promega) and normalized to the protein content of lysates.

Gene expression analysis

Total RNA was purified using RNeasy minicolumns and on-column ribonuclease-free deoxyribonuclease treatment (Qiagen Inc.). Integrity of RNA was determined using the RNA Nano Lab chip kit and Bioanalyzer (Agilent). Reverse transcription and SYBR Green real-time PCR assays were performed exactly as described previously (Giesy *et al.* 2012). Primers are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. mRNA data were analyzed using a relative standard curve based on serial two-fold dilutions of pooled cDNA prepared from the liver. Unknown sample expression was determined from the standard curve, adjusted for expression of the invariant control gene *B2M*, and reported as a fold difference.

Western blot analysis

Plasma samples (2.5 µL of a 1:6 dilution) were mixed with equal volumes of 2× Laemmli buffer containing dithiothreitol and mercaptoethanol and boiled for 10 min.

They were electrophoresed on 13% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience, Keene, NH, USA). The membranes were blocked in Tris-buffered saline with Tween-20 (TBST; 0.05 M Tris, pH 7.4, 0.2 M NaCl, 0.1% Tween-20) containing 3% w/v nonfat dried skim milk (NFM) for 1 h at room temperature and incubated with 1:1000 dilution of bovine insulin-like growth factor-binding protein 2 (IGFBP2) antiserum (#06-107, Millipore) in the blocking buffer for 2 h at room temperature. After washes in TBST, membranes were incubated with 1:20,000 IRDye 800 goat anti-rabbit secondary antibody (LI-COR Biotechnology, Lincoln, NE, USA) in TBST with 3% NFM for 1 h at room temperature. Signals were quantified with the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) using the 800-nm channel.

Statistical analysis

Data were analyzed by the MIXED procedure of SAS (SAS Institute, Raleigh, NC, USA). Data obtained during the transition period were analyzed with a model accounting for physiological stage (LP or EL) as a fixed effect and cow as a random effect. For the infusion study, data collected over time were analyzed with a model accounting for treatment (Control or hLeptin), time and their interaction as fixed effects and cow as a random effect. All other variables were analyzed with a model accounting for treatment (Control or hLeptin) as fixed effects and cow as a random effect. A covariate corresponding to data collected prior to infusion (i.e., between days 6 and 8 of EL) was included in the statistical models used for the infusion period only when significant. The level of significance was set at $P < 0.05$ for main effects and $P < 0.10$ for interactions.

Results

Changes in energy-related variables during the transition from late pregnancy to early lactation

Cows were studied in LP between days -32 and -29 and again in EL between days 6 and 8. Energy balance shifted from a surplus of 12 Mcal in LP to a deficit of 15 Mcal in EL (Table 1; $P < 0.01$). This shift reflected increased energy output in the form of milk in the absence of a compensatory increase in energy intake. The nutritional deficit of EL was associated with reductions of 18% in plasma glucose, 62% in liver glycogen, and 25% in the

Table 1 Effect of the transition from LP to EL on whole-animal and metabolic variables.

| | Physiological state ^a | | S.E.M. | P-value ^b |
|---|----------------------------------|-------|--------|----------------------|
| | LP | EL | | |
| Whole animal | | | | |
| Body weight, kg | 648 | 575 | 13 | <0.01 |
| Dry matter intake, kg/day | 15.6 | 15.5 | 0.8 | NS |
| Energy intake, Mcal/day | 22.4 | 24.2 | 1.2 | NS |
| Energy balance, Mcal/day | 12.1 | -15.2 | 1.5 | <0.01 |
| Glucose response area ^c , mg glucose/dL × min | 1182 | 878 | 55 | <0.01 |
| Plasma variables | | | | |
| Glucose, mg/dL | 66 | 54 | 1 | <0.01 |
| Free fatty acids, μM | 108 | 386 | 43 | <0.01 |
| Insulin, ng/mL | 0.26 | 0.08 | 0.01 | <0.01 |
| IGF1, ng/mL | 193 | 56 | 11 | <0.01 |
| T ₄ , ng/mL | 5.13 | 1.75 | 0.23 | <0.01 |
| T ₃ , ng/mL | 1.58 | 0.95 | 0.06 | <0.01 |
| Leptin, ng/mL | 3.1 | 2.3 | 0.1 | <0.01 |
| Cortisol, ng/mL | 0.30 | 0.37 | 0.05 | NS |
| Liver | | | | |
| Triglycerides, % wet weight | 1.4 | 7.2 | 1.0 | <0.01 |
| Glycogen, % wet weight | 2.72 | 0.85 | 0.19 | <0.0001 |

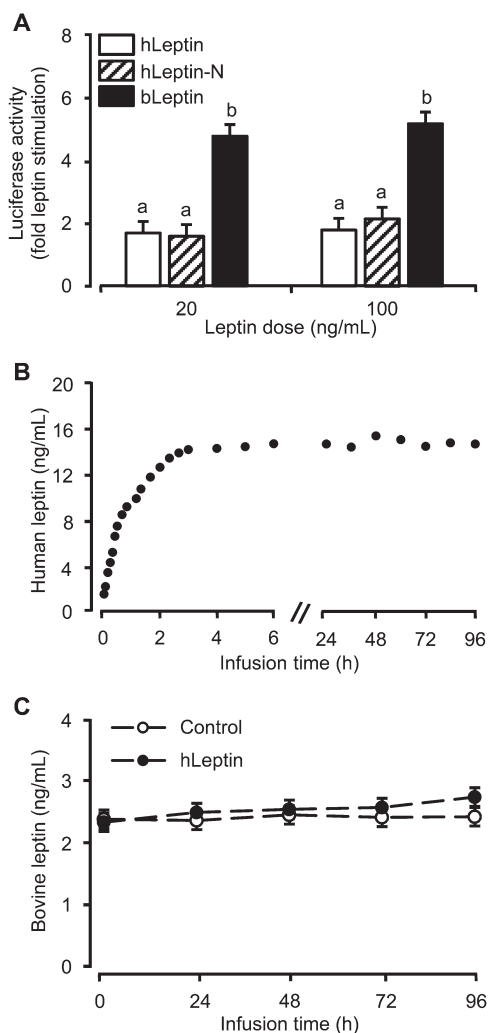
^aDairy cows ($n = 10$) were studied over a 4-day period in late pregnancy (LP) (day -32 to -29 relative to parturition on day 0) and again over a 3-day period in early lactation (EL) (days 6-8); ^bType I error probability for physiological state; NS, nonsignificant; ^cGlucose response area during the insulin tolerance test calculated between time 0 (time of insulin bolus) and 180 min and corrected for the difference in baseline glucose concentration.

glucose response area during the insulin tolerance test ($P < 0.01$ or less). By contrast, the plasma concentration of free fatty acids and the liver triglyceride content were, respectively, 3.6- and 6-fold higher in EL than in LP ($P < 0.01$). These data illustrate the precarious glucose economy of EL and adaptations preserving its availability for mammary use (e.g., attenuation of insulin-dependent use and increased reliance on mobilized lipids).

The transition from LP to EL led to reductions in plasma insulin, IGF1, thyroid hormones, and leptin ($P < 0.01$). Reduced plasma concentrations of the first three of these hormones have been shown to drive adaptive metabolism in early lactating dairy cows (Bauman & Vernon 1993, Bell 1995, Boisclair *et al.* 2006), but the contribution of reduced plasma leptin remains unknown.

Effect of increased plasma leptin on the whole animal energy budget in early lactation

To address the role of leptin in EL, we sought to reverse its reduction with exogenous administration of hLeptin.

**Figure 1**

Bioactivity and plasma profile of human leptin in cattle. (A) HEK293 cells were transfected in duplicate with plasmid bOBR_h encoding the full-length bLeptin receptor and the STAT3 luciferase reporter plasmid p4xM67-TK-Luc. Transfected cells were incubated for 24 h in serum-free media or in serum-free media supplemented with 20 or 100 ng/mL of human leptin (hLeptin) obtained from either Eli Lilly (hLeptin) or from the National Hormone and Peptide Program (hLeptin-N) or bovine leptin (bLeptin). Firefly luciferase was measured in cell extracts, corrected for protein concentration, and expressed as a fold of luciferase expression in untreated transfected cells. Each bar represents the mean \pm S.E.M. of two replicates. For each concentration, bars with different letters differ at $P < 0.05$. Similar results were obtained in a second experiment. (B) The plasma concentration of human leptin in dairy cows receiving a constant intravenous infusion of recombinant human leptin (hLeptin). The infusion started on day 8 of lactation and lasted for 96 consecutive hours. The plasma concentration was measured with a human leptin RIA devoid of cross-reactivity toward bovine leptin. Each point represents the mean of hLeptin concentration ($n=5$) and has a pooled standard error of 1.0 ng/mL. (C) The plasma concentration of bovine leptin was measured in dairy cows receiving a constant intravenous infusion of saline (Control) or recombinant hLeptin. The infusion started on day 8 of lactation and lasted for 96 consecutive hours. The plasma concentration was measured with a bovine leptin RIA devoid of cross-reactivity toward human leptin. Each point represents the mean \pm S.E.M. of the plasma concentration of bovine leptin ($n=5$).

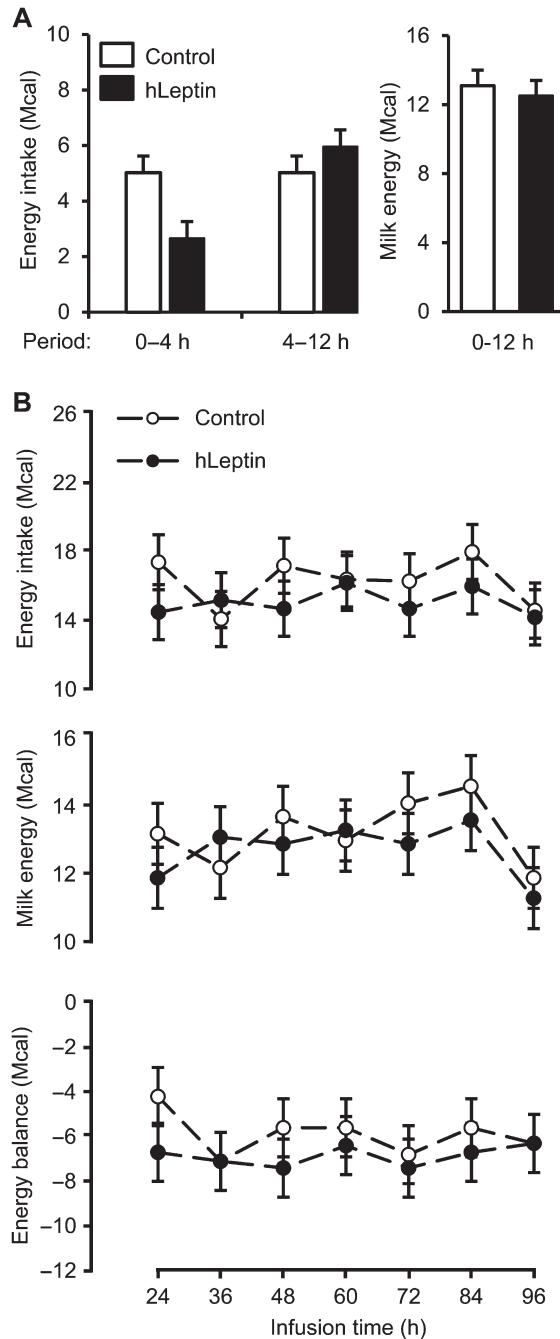
As a first step, the ability of hLeptin to activate the bLeptin receptor was estimated in HEK293 cells cotransfected with plasmids encoding the full-length bLeptin receptor and a STAT3-dependent luciferase gene. Cells were treated for 24 h with 0, 20, or 100 ng/mL of either hLeptin or bLeptin, and luciferase activity was measured (Fig. 1A). At the 20 ng/mL dose, hLeptin stimulated the luciferase activity 1.9-fold, but less than the 5-fold activation obtained with bLeptin ($P < 0.05$); hLeptin-N, a human leptin produced by a different supplier, gave identical results. No further stimulation was seen with any of these hormones when used at the 100 ng/mL dose. These *in vitro* data show that human leptin activates the bLeptin receptor but with less potency than its bovine counterpart.

Next, cows were assigned to receive on day 8 of lactation a constant intravenous infusion of saline (Control) or hLeptin for 96 h. In the hLeptin group, human leptin rose to a steady-state concentration of 14.0 ± 1.0 ng/mL within 2.3 h of infusion, consistent with a calculated half-life of 28.0 ± 5.0 min in the vascular compartment (Fig. 1B). The plasma concentration of bLeptin was not affected by the hLeptin infusion and did not differ between the Control and hLeptin groups during the infusion period (Fig. 1C).

Voluntary feed intake was recorded over two time intervals during the first 12 h of infusion and every 12 h thereafter. Feed intake expressed on an energy basis appeared lower after 4 h of hLeptin infusion, but this numerical reduction disappeared during the following 8 h (Fig. 2A) and never reached significance over the next 84 h (Fig. 2B). The hLeptin infusion did not alter milk secretion over the first 12 h of infusion or at later times, whether expressed on an energy basis (Fig. 2A and B) or on a volume and composition basis (Supplementary Table 2). Similarly, the energy balance calculated from these primary variables was not affected by the hLeptin treatment when assessed every 12 h or over the entire infusion period (Fig. 2B and results not shown). Therefore, the periparturient reduction in plasma leptin is not necessary to sustain the appetite and rising milk production of early lactating dairy cows.

Effect of increased plasma leptin on glucose and lipid metabolism in the liver and the whole animal

Leptin therapy stimulates glucose utilization in various rodent models (Kamohara *et al.* 1997, Chinookoswong *et al.* 1999), but whether it is capable of similar actions in the insulin-resistant, early lactating dairy cow is unknown. The hLeptin infusion caused a 9% reduction in plasma glucose (Table 2, $P < 0.01$) despite no effects on the hepatic

**Figure 2**

Effect of leptin infusion in early lactation on energy-related variables. Dairy cows received a constant intravenous infusion of either saline (Control) or human leptin (hLeptin) starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Voluntary feed intake and milk output were measured over specific periods during the first day of infusion and expressed on an energy basis. Each bar represents the mean \pm S.E.M. of the indicated variable ($n=5$). (B) Dry matter intake, milk yield, and milk composition were measured every 12 h. These data were used to calculate energy intake, milk energy output, and net energy balance as described in the "Materials and methods" section. Each data point represents the mean \pm S.E.M. of the indicated variable ($n=5$).

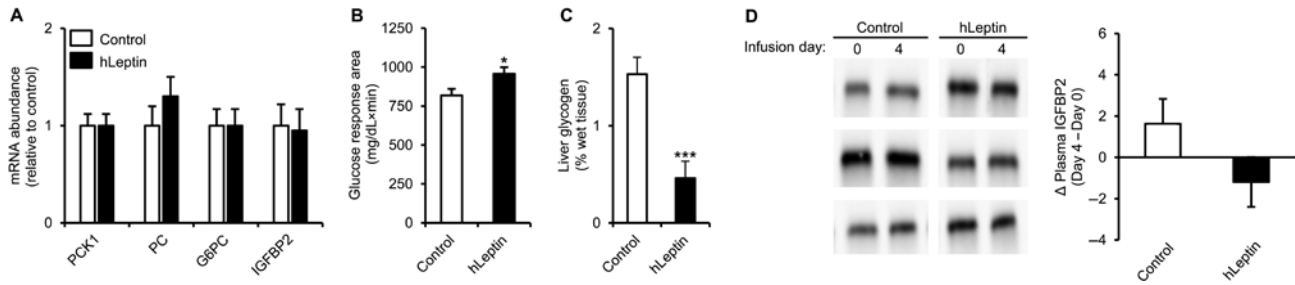
Table 2 Effect of hLeptin infusion in early lactation on plasma variables.

| | Infusion ^a | | S.E.M. | P-value ^b |
|---------------------------|-----------------------|---------|--------|----------------------|
| | Control | hLeptin | | |
| Plasma metabolites | | | | |
| Glucose, mg/dL | 55.3 | 50.4 | 0.8 | <0.01 |
| Free fatty acids, μ M | 349 | 408 | 62 | NS |
| Plasma hormones | | | | |
| Insulin, ng/mL | 0.12 | 0.09 | 0.02 | NS |
| Cortisol, ng/mL | 0.21 | 0.19 | 0.03 | NS |
| GH, ng/mL | 6.0 | 7.6 | 0.5 | 0.04 |
| IGF1, ng/mL | 67 | 53 | 5 | NS |
| LH, ng/mL | 0.60 | 0.61 | 0.06 | NS |

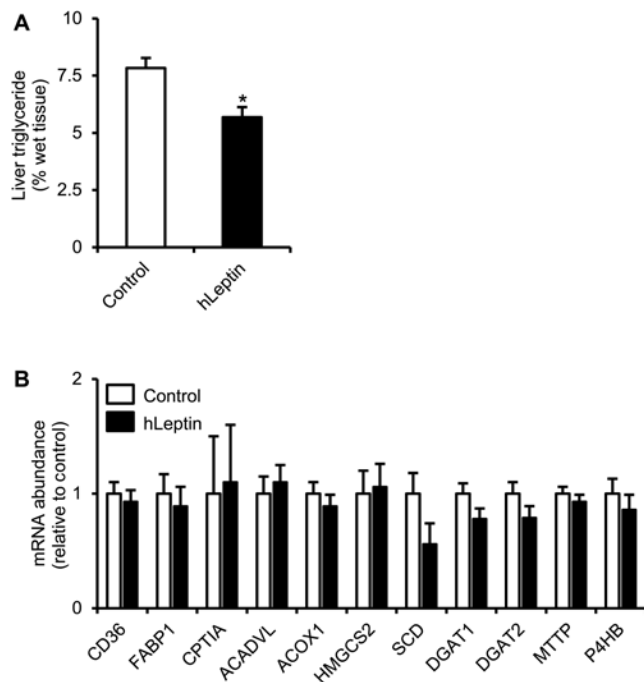
^aDairy cows received continuous intravenous infusion of saline (control) or hLeptin between days 8 and 12 of lactation ($n=5$ cows per treatment); ^bType I error probability for physiological state; NS, nonsignificant.

mRNA abundance of the gluconeogenic enzymes *PCK1*, *PC*, and *G6PC* (Fig. 3A). The hLeptin infusion increased the glucose response area by 17% during the insulin tolerance test (Fig. 3B, $P<0.05$) in absence of any effects on plasma insulin (Table 2). Finally, hepatic glycogen content was 73% lower in hLeptin than in control cows (Fig. 3C, $P<0.001$), suggesting that increased plasma leptin in EL leads to a more precarious glucose economy. This effect reflected a substantial recovery of hepatic glycogen over the treatment period in Control cows (i.e., from 0.85% wet liver weight on day 8 to 1.53% on day 12) vs continued depression in the hLeptin-treated cows (i.e., 0.46% wet liver weight on day 12). In leptin-treated mice, enhanced insulin-mediated glucose metabolism has been linked to increased IGF2 production (Hedbacker *et al.* 2010). However, the hLeptin infusion did not increase hepatic *IGFBP2* mRNA abundance by the end of treatment (Fig. 3A) or induce a positive change in plasma IGF2 over the treatment period (Fig. 3D). Overall, these data suggest that the reduced plasma leptin of EL promotes glucose conservation by attenuating insulin action.

Leptin therapy reduces hepatic lipid accumulation in various rodent models (Levin *et al.* 1996, Cohen *et al.* 2002). To determine whether this property is conserved during the pro-steatotic period of EL in dairy cows, liver triglycerides were measured. Hepatic triglyceride content was 28% lower in the hLeptin than in saline-infused cows (Fig. 4A, $P<0.05$). This anti-steatotic effect occurred in the absence of changes in the hepatic expression of genes involved in the transport of free fatty acids across membranes (*CD36*, *FABP1*, *CPT1A*), oxidation (*ACADVL*, *ACOX1*), ketogenesis (*HMGCS2*), or assembly of fatty acids into triglyceride and export as VLDL (*SCD*, *DGAT1*, *DGAT2*, *MTTP*, *P4HB*) (Fig. 4B). Moreover, hLeptin did

**Figure 3**

Effect of leptin infusion in early lactation on indicators of glucose metabolism. Dairy cows received a constant intravenous infusion of either saline (Control) or human leptin (hLeptin) starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Total RNA was obtained from liver biopsies taken at the end of infusion and analyzed by quantitative real-time PCR for expression of the indicated genes. For each gene, the expression of the indicated mRNA is relative to control, with each bar representing the mean \pm S.E.M. of five cows. (B) The plasma glucose response area was calculated during the insulin tolerance test performed on day 3 of infusion as described in the "Materials and methods" section. Each bar represents the mean \pm S.E.M. of five cows. * $P < 0.05$. (C) Glycogen content was measured on liver biopsies obtained at the end of infusion. Each bar represents the mean \pm S.E.M. of five cows. *** $P < 0.001$. (D) Plasma samples collected immediately before (day 0) and at the end of infusion (day 4) were analyzed on a single gel for IGFBP2 abundance by Western immunoblotting. Panels on the left show three cows for each treatment. IGFBP2 signals were quantified with the LI-COR Odyssey infrared imaging system, and the change in plasma IGFBP2 abundance over the infusion period (Δ Plasma IGFBP2) was calculated for each cow. Bars on the right represent the mean change in plasma IGFBP2 abundance, with each bar representing the mean \pm S.E.M. of five cows.

**Figure 4**

Effect of leptin infusion in early lactation on liver triglyceride levels. Dairy cows received a constant intravenous infusion of either saline (Control) or human leptin (hLeptin) starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Liver biopsies were obtained at the end of infusion and analyzed for triglyceride content. Each bar represents the mean \pm S.E.M. of five cows. * $P < 0.05$. (B) Total RNA was obtained from liver biopsies taken at the end of infusion and analyzed by quantitative real-time PCR for expression of the indicated genes. For each gene, the expression of the indicated mRNA is relative to control, with each bar representing the mean \pm S.E.M. of five cows.

not alter plasma free fatty acids (Table 2), ruling out a secondary effect through reduced lipid mobilization from adipose tissue. Overall, these data suggest that the reduction of plasma leptin around parturition exacerbates hepatic lipid accumulation in EL.

Effect of increased plasma leptin on plasma thyroid hormones

Energy-deficient animals, including dairy cows in EL (Table 1), rely on a depressed hypothalamic–pituitary–thyroid axis to reduce energy consumption in tissues such as skeletal muscle (Mullur *et al.* 2014, Rosenbaum & Leibel 2014). Consistent with leptin dependence of this axis in EL, cows infused with hLeptin experienced increases of ~45% in the plasma concentrations of both T_4 and T_3 (Fig. 5A, $P < 0.01$). In the case of plasma T_3 , this stimulation led to a near complete correction to concentrations prevailing during the energy sufficient state of LP (i.e., 1.32 ng/mL in EL cows treated with hLeptin vs 1.58 ng/mL in LP), whereas plasma levels remained depressed in Control cows (0.91 ng/mL); similar observations were made for plasma T_4 . Consistent with promotion of metabolic rate by thyroid hormones, the hLeptin-treated cows lost more weight than Control cows during the infusion period (24 ± 1.7 vs 18 ± 1.7 kg, $P < 0.02$). By contrast, levels of plasma cortisol, which remained unchanged between LP and EL, were unaffected by the hLeptin infusion (Tables 1 and 2). These data implicate plasma

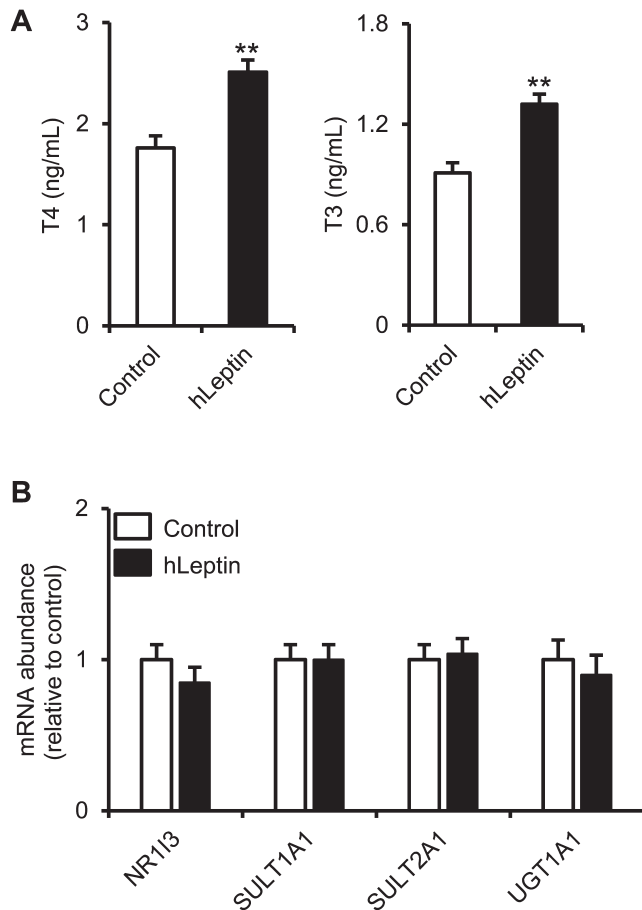


Figure 5

Effect of leptin infusion in early lactation on the plasma concentration of thyroid hormones. Dairy cows received a constant intravenous infusion of either saline (Control) or human leptin (hLeptin) starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Plasma samples were obtained over the last 8 h of the infusion period and analyzed for the concentration of T₄ and T₃. For each hormone, each bar represents the mean \pm s.e.m. of five cows. ** $P < 0.01$. (B) Total RNA was obtained from liver biopsies taken at the end of infusion and analyzed by quantitative real-time PCR for expression of the indicated genes. For each gene, the expression of the indicated mRNA is relative to control, with each bar representing the mean \pm s.e.m. of five cows.

leptin as a determinant of plasma thyroid hormone levels and energy conservation in EL dairy cows.

Increased removal of thyroid hormones through hepatic sulfotransferases and glucuronidases contributes to their reduced concentrations in fasted rodents (Maglich *et al.* 2004, Vella *et al.* 2011). The hLeptin infusion, however, had no effect on the hepatic expression of the constitutive androstane receptor *NR1I3* or its target sulfotransferase and glucuronidase genes *SULT1A1*, *SULT2A1*, and *UGT1A1* (Fig. 5B). Accordingly, the positive effects of hLeptin on plasma thyroid hormones in early

lactating dairy cows are unlikely to involve reduced hepatic removal.

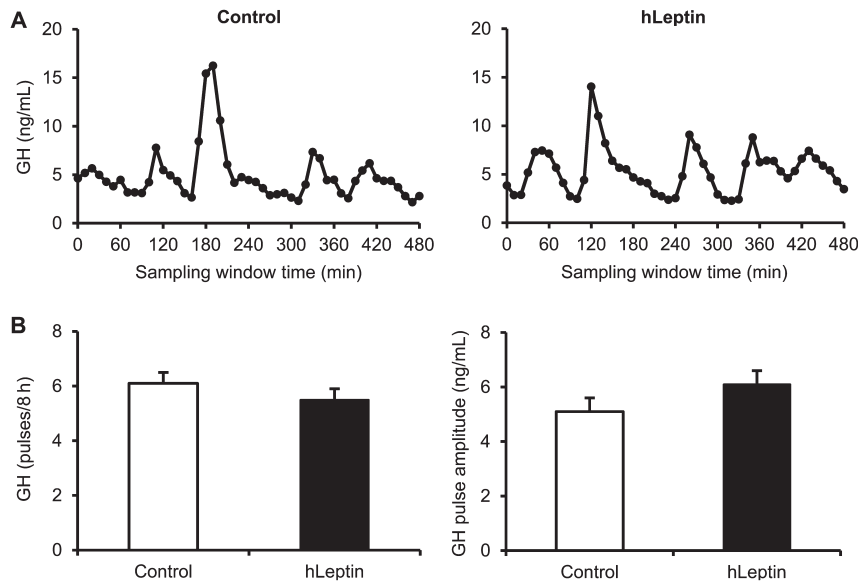
Effect of increased plasma leptin on secretion of GH and LH

In dairy cows, the energy insufficiency of EL increases plasma GH and has the opposite effect on pulsatile LH secretion (Butler 2000, Boisclair *et al.* 2006). To determine whether exogenous leptin can reverse these effects, blood samples were obtained every 10 min over an 8-h period on the last day of infusion. hLeptin infusion increased rather than decreased the average plasma GH concentration (Table 2, $P < 0.04$), an effect that occurred without measurable differences in either GH pulse frequency or amplitude (Fig. 6) and in the absence of any increase in plasma IGF1 (Table 2). The hLeptin infusion was without effects on the average plasma LH concentration or other secretory variables (Table 2 and Fig. 7). These data do not support a causal role for the periparturient reduction of plasma leptin in increasing GH secretion or in suppressing LH secretion in early lactating dairy cows.

Discussion

In the modern dairy cow, milk production increases within days of parturition to near maximum levels. As a consequence, the mammary gland consumes as much energy on day 4 of lactation as the sum of all other maternal tissues and 4 times as much energy as the gravid uterus in LP (Bell 1995). The mammary demand for glucose is similarly extensive, with 85% of the whole animal supply devoted to lactose synthesis (Bell 1995). These nutritional challenges are compounded by the inability of dairy cows, unlike rodents, to increase voluntary feed intake in proportion to milk yield in EL (Bell 1995, Johnson *et al.* 2001, Vernon *et al.* 2002). The consequent energy insufficiency causes a reduction in leptin production even before significant depletion of fat depots occurs (Block *et al.* 2001, Reist *et al.* 2003, Janovick *et al.* 2011). We now provide evidence that this reduction in plasma leptin contributes to the adaptive metabolism of the early lactating dairy cow.

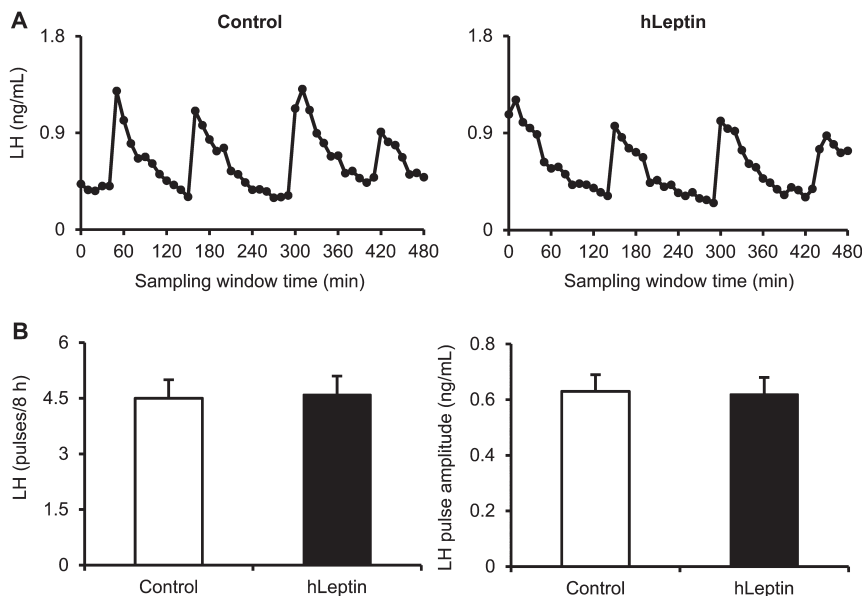
Virtually, all *in vivo* studies of leptin actions in ruminants have involved intracerebroventricular (ICV) administration (Henry *et al.* 1999, Morrison *et al.* 2001, Miller *et al.* 2002, Foskolos *et al.* 2015). Although these studies provide unambiguous evidence of leptin actions in ruminants, including anorexic actions,

**Figure 6**

Effect of leptin infusion in early lactation on the profile of plasma GH. Dairy cows received a constant intravenous infusion of either saline (Control) or hLeptin starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Plasma samples were obtained every 10 min over the last 8 h of the infusion period and analyzed for the concentration of GH. A representative cow is shown for each treatment. (B) The number of GH pulses and their amplitude were quantified as described in the "Materials and methods" section. Bars in each graph represent the mean \pm S.E.M. of five cows.

they have two significant limitations. First, none of these studies were conducted in lactating animals. Second, as recently discussed (Foskolos *et al.* 2015), these studies exposed neuronal centers to supraphysiological leptin concentrations and, therefore, provided little insight into the consequences of normal variation in plasma leptin. As an alternative, we countered the periparturient leptin reduction by infusing hLeptin into the vascular compartment. This approach produced steady-state plasma levels of 14 ng/mL for hLeptin, which, after correcting for its activity in the luciferase bioassay, raised the total effective leptin concentration to approximately 8 ng/mL (i.e., 14 ng/mL of human leptin \times 38% + 2.6 ng/mL

of bovine leptin), well within the range of concentrations reported in late pregnant dairy cows (Block *et al.* 2001, Janovick *et al.* 2011, Reist *et al.* 2003). This elevation did not cause a significant reduction in energy intake at any time during the infusion period and had no impact on the milk energy output. One interpretation of these data is that voluntary feed intake becomes unresponsive to variation in plasma leptin during the energy deficit of EL, an idea supported by the failure of ICV leptin to reduce feed intake in severely feed-restricted sheep (Henry *et al.* 2001, Morrison *et al.* 2001). If leptin resistance exists in early lactating dairy cows, reduced plasma leptin may not lead to higher voluntary feed intake.

**Figure 7**

Effect of leptin infusion in early lactation on the profile of plasma LH. Dairy cows received a constant intravenous infusion of either saline (Control) or hLeptin starting on day 8 of lactation and lasting for 96 consecutive hours. (A) Plasma samples were obtained every 10 min over the last 8 h of the infusion period and analyzed for the concentration of LH. A representative cow is shown for each treatment. (B) The number of LH pulses and their amplitude were quantified as described in the "Materials and methods" section. Bars in each graph represent the mean \pm S.E.M. of five cows.

Our results illustrate the dynamic changes in glucose and lipid utilization during the transition from LP to EL and provide evidence for leptin regulation of these adaptations. With respect to glucose, low plasma insulin in EL combines with insulin resistance to limit glucose use by nonmammary tissue, safeguarding the available supply for lactose synthesis (Bell 1995, Boisclair *et al.* 2006). Correcting the leptin deficit of EL increased insulin-mediated glucose disposal and led to a more precarious glucose economy, as reflected by reduced plasma glucose and liver glycogen. We considered some of the mechanisms invoked to explain the positive actions of leptin on insulin action and glucose disposal. First, we asked whether leptin stimulated liver-derived IGFBP2, a protein reported to alleviate insulin resistance in the leptin-deficient *ob/ob* mice (Hedbacker *et al.* 2010). Second, we examined whether leptin repressed GH secretion. This second possibility was of interest because GH secretion is increased in early lactating dairy cows and antagonizes insulin-mediated glucose disposal (Sechen *et al.* 1990, Block *et al.* 2001, Rhoads *et al.* 2004). Moreover, ICV leptin prevented the increased GH secretion seen in pair-fed intact rams and in fasted adult ewes (Blache *et al.* 2000, Henry *et al.* 2004). However, neither mechanism explained the effect of leptin on the glucose economy as the hLeptin infusion failed to increase liver IGFBP2 expression and increased rather than decreased plasma GH concentration. The more precarious glucose economy with hLeptin treatment could also reflect the effects that could not be measured, including increased glucose uptake in skeletal muscle following activation of the autonomic nervous system (Minokoshi *et al.* 2002) and leptin stimulation of immune cell activity and associated glucose utilization (Palsson-McDermott & O'Neill 2013, Naylor & Petri 2016). Irrespective of the mechanism involved, however, our data point to a reduction in plasma leptin as one of the signals promoting glucose conservation during the transition from LP to EL.

Liver steatosis is a common feature of leptin-deficient rodents and was specifically attributed to reduced lipid oxidation by pair-feeding these animals to wild-type controls (Cohen *et al.* 2002, Prieur *et al.* 2008). Leptin therapy of these animals corrected the oxidative defect and the associated steatosis through repression of the hepatic fatty acid desaturase *SCD1* (Cohen *et al.* 2002). The nature of liver steatosis differs in early lactating dairy cows, in that it is caused by excessive uptake of circulating free fatty acids, arising secondarily to their accelerated mobilization from adipose tissue (Drackley *et al.* 2001). Leptin remained effective in combating steatosis in

these animals, reducing liver lipids by 28% over a mere 4 days of treatment. However, we found no evidence that hLeptin altered the hepatic expression of *SCD1*, the bovine ortholog of *SCD1*, or of any other genes involved in the uptake, oxidation, or export of free fatty acids, suggesting a posttranscriptional effect. In this context, lipophagy has been recently implicated in the liver in the delivery of fatty acids from lipid droplets to the oxidative machinery (Singh *et al.* 2009). Lipophagy is not only engaged posttranslationally but also dependent on adequate thyroid hormone signaling (Singh *et al.* 2009, Sinha *et al.* 2012). Future work is needed to determine whether the anti-steatotic effects of hLeptin in early lactating dairy cows represent the activation of lipophagy in response to increased plasma thyroid hormones.

Circulating thyroid hormones are also known as rheostat of basal metabolism, particularly in cardiac and skeletal muscles (Lebon *et al.* 2001, Mullur *et al.* 2014). Accordingly, the reduction in circulating thyroid hormones in early lactating dairy cows has been viewed as an energy sparing mechanism (Vernon *et al.* 2002). This view is supported by increased energy expenditure and excessive weight loss in cows treated with exogenous thyroid hormones (Thomas 1953, Knight *et al.* 2004). The hLeptin infusion partially corrected the thyroid hormone deficit and exacerbated the loss of body weight without impacting milk production, suggesting an increase in a nonmammary component of energy expenditure. Our data resemble those obtained in humans sustaining a 10% reduction in body weight (Rosenbaum *et al.* 2005). These individuals experience reduced concentrations of thyroid hormones and energy expenditure and correction of both defects when treated peripherally with a low dose of leptin (Rosenbaum *et al.* 2005, Rosenbaum & Leibel 2014). Interestingly, skeletal muscle was identified as the effector tissue for the opposing effects of weight loss and leptin on energy expenditure (Baldwin *et al.* 2011). Overall, our data implicate plasma leptin as a major determinant of circulating thyroid hormones and energy sparing in transition dairy cows.

The modulation of circulating thyroid hormones by leptin in rodents is the sum of reciprocal effects on production and removal. On the production side, leptin activates hypophysiotropic thyrotrophin-releasing hormone (TRH) neurons directly via its receptor, as well as indirectly by modulating the production of the neuropeptides α -melanocyte-stimulating hormone (α -MSH) and neuropeptide Y (NPY) in the arcuate nucleus (Fekete & Lechan 2014). Similar actions appear possible in dairy cows as we have recently showed that ICV infusion

of a leptin antagonist reduced plasma thyroid hormone in the sheep (Foskolos *et al.* 2015), a closely related ruminant, whereas ICV infusion of an α -MSH analog had the opposite effect (RA Ehrhardt and YR Boisclair, unpublished observations). With respect to removal, the liver contributes to the reduction of circulating thyroid hormones in fasted rodents through an increased expression of sulfotransferases and glucuronidases (Maglich *et al.* 2004, Vella *et al.* 2011, de Vries *et al.* 2014). In our study, however, we found no evidence that the positive effects of hLeptin on plasma thyroid hormones involve repression of liver sulfotransferases or glucuronidases.

Early lactating dairy cows often suffer from delayed or failed first ovulation and decreased fertility (Butler 2000). These defects have been attributed in part to the suppression of pulsatile LH secretion by the coexisting energy deficit. Given the negative association between plasma leptin and energy balance, hypoleptinemia in EL has been viewed as a candidate mediator of this effect. This is supported by positive associations among plasma leptin, LH pulsatility, and the time to first estrus in early lactating dairy cows (Kadokawa *et al.* 2000, Liefers *et al.* 2003), and by functional data showing leptin rescue of LH pulsatility in undernourished rams, ewes, and beef cows (Nagatani *et al.* 2000, Henry *et al.* 2001, Amstalden *et al.* 2002). These functional data, however, were obtained in gonadectomized animals with amplified sensitivity of the gonadotrophin-releasing hormone (GnRH) pulse generator to the repressive effects of energy insufficiency. As shown by our results, energy-deficient, early lactating dairy cows retain significant LH pulsatility, and this is unaltered by infusion of hLeptin.

In summary, we show that some of the metabolic adaptations of EL (i.e., reduced insulin-mediated glucose disposal, reduced plasma thyroid hormones, and hepatic lipid accumulation) are attenuated by restoration of plasma leptin to levels prevailing in LP. These data suggest that the reduction of plasma leptin between LP and EL is one of the signals contributing to these adaptations in the modern dairy cow. Future work is needed in dairy cows to determine whether leptin regulation of thyroid hormone production underpins its effects on liver lipids and whether skeletal muscle is the main effector tissue of leptin actions on insulin response and energy sparing.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0031>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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