Adiponectin Deficit During the Precarious Glucose Economy of Early Lactation in Dairy Cows

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In rodents and primates, insulin resistance develops during pregnancy and fades after parturition. In contrast, dairy cows and other ruminants maintain insulin resistance in early lactation (EL). This adaptation favors mammary glucose uptake, an insulin-independent process, at a time when the glucose supply is scarce. Reduction in circulating levels of the insulin-sensitizing hormone adiponectin promotes insulin resistance in other species, but whether it contributes to insulin resistance in EL dairy cows is unknown. To address this question, plasma adiponectin was measured in high-yielding dairy cows during the transition from late pregnancy (LP) to EL. Plasma adiponectin varied in quadratic fashion with the highest levels in LP, a maximal reduction of 45% on the day after parturition and a progressive return to LP values over the next 8 wk. Adiponectin circulated nearly exclusively in high molecular weight complexes in LP, and this distribution remained unaffected in EL. The reduction of plasma adiponectin in EL occurred without changes in adiponectin mRNA in adipose tissue but was associated with repression of the expression of proteins associated with the endoplasmic reticulum and involved in assembly of adiponectin oligomers. Finally, EL increased the expression of the adiponectin receptor 1 in muscle and adiponectin receptor 2 in liver but had no effect on the expression of these receptors in adipose tissue and in the mammary gland. These data suggest that reduced plasma adiponectin belongs to the subset of hormonal adaptations in EL dairy cows facilitating mammary glucose uptake via promotion of insulin resistance. (***Endocrinology*** **153: 5834–5844, 2012)**

Glucose is the primary fuel of fetal life and is translocated from the maternal circulation by the placenta in an insulin-independent manner (1). This process is facilitated by the development of maternal insulin resistance over the course of pregnancy (2–4). This adaptation fades after parturition in rodents and primates with the consequence that insulin action is normalized during the ensuing lactation (3, 5). In dairy cows and other ruminants, however, insulin resistance is preserved and even increased in early lactation (EL) (1, 6, 7). Maintenance of this adaptation relates in part to the inability of these animals to increase voluntary feed intake sufficiently after parturition to cover the nutrient requirements of lactation and to the consequent severe energy insufficiency (6, 8, 9). In this context, insulin resistance facilitates the biosynthetic activities of the mammary gland by preserving available glucose for lactose synthesis and by promoting mobilization of endogenous reserves (6, 8).

Insulin resistance in EL cows is mediated in part by endocrine mechanisms, such as increased growth hormone secretion (1, 6, 9). One recently discovered modulator of insulin action that has not been considered in dairy

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Abbreviations: Ad_{N14}, 14 Amino-terminal residues of bovine adiponectin; AdipoR, adiponectin receptor; AMPK, AMP-activated kinase; CP, crude protein; DM, dry matter; DsbA-L, disulfide-bond A oxidoreductase-like protein; EL, early lactation; ER, endoplasmic reticulum; Ero1-Lα, ERO1-like protein α; Erp44, ER protein 44; GGA1, Golgi-localizing γ adaptin ear homology domain ARF-binding protein;Grp78, glucose-regulated protein, 78 kDa; HMW, high MW; LMW, low molecular weight; LP, late pregnancy; MMW, medium MW; NEFA, nonesterified fatty acids; NE_L, net energy of lactation; NFM, nonfat dried skim milk; Pdia6, protein disulfide isomerase family A, member 6; PPAR, peroxisome proliferator-activated receptor; TBST, Tris-buffered saline with Tween 20; TMR, total mixed ration; WAT, white adipose tissue.

cattle is adiponectin, a protein hormone produced predominantly by white adipose tissue (WAT) (10–12). In mice and primates, adiponectin circulates in low molecular weight (LMW), medium MW (MMW), and high MW (HMW) complexes containing 3, 6, or 18 or more adiponectin monomers (10, 12, 13). Mice lacking adiponectin have reduced insulin action under basal conditions and develop greater insulin resistance when receiving a high-fat/high-sucrose diet (14, 15). Remarkably, adiponectin overexpression in ob/ob mice normalizes glycemia and insulin action despite promoting further obesity (16). These insulin-sensitizing properties of adiponectin are corroborated by human studies: the insulin resistance of obesity and type 2 diabetes is associated with reduced plasma adiponectin, whereas this reduction is reversed by interventions improving insulin action, such as weight loss and therapy with peroxisome proliferator-activated receptor (PPAR)γ agonists (17–19).

These data led us to hypothesize that plasma adiponectin is decreased in dairy cows during the persistence of insulin resistance after parturition. In mice and humans, the insulin resistance of obesity and type 2 diabetes tracks with reduced HMW adiponectin (20, 21), but whether the same association occurs during the persistence of insulin resistance in EL is unknown. Accordingly, we determined whether EL caused an alteration in the MW distribution of circulating adiponectin. Finally, adiponectin signals via two receptors known as adiponectin receptor (AdipoR) 1 and AdipoR2 (10, 22). Therefore, we examined the effects of EL on expression of these receptors in liver, skeletal muscle, and adipose tissue where glucose uptake is insulin dependent and in the mammary gland where it is not. We found that plasma adiponectin is lower in EL than in late pregnancy (LP), and it circulates nearly exclusively in HMW complexes in both physiological states. The reduction of plasma adiponectin in EL occurred in the absence of any change in adiponectin mRNA expression in WAT, but rather was associated with a reduction in expression of a subset of endoplasmic reticulum (ER)-associated proteins involved in adiponectin assembly. Finally, AdipoR expression was induced after parturition but only in liver and muscle, the two tissues responsible for the bulk of lipid oxidation in EL.

Materials and Methods

Animals and design

All experiments were performed with mature Holstein cows at Cornell University using a set of standard procedures. In brief, animals were housed in individual stalls and fed unlimited amounts of total mixed rations (TMR) formulated for high-yielding dairy cows in LP or EL. Milking frequency in EL was thrice daily for the first experiment and twice daily for all other experiments. Plasma was prepared from collected blood samples by addition of sodium heparin (15 IU/ml) and centrifugation. Tissues were collected after local anesthesia using a biopsy tool for liver and mammary gland and dissection for tail head WAT and semitendinosus muscle (23–25). Tissues were frozen immediately in liquid nitrogen and stored at −80 C until used for RNA analyses. The net energy balance of each animal was calculated as the difference between ingested energy and energy expenditures (maintenance and pregnancy during LP and maintenance and milk energy during EL) (23, 25, 26). All animal procedures were performed with approval of the Institutional Animal Care and Use Committee.

A first experiment covering the last 35 d of pregnancy and first 56 d of lactation was used to describe the plasma adiponectin profile (23). The nutrient composition of the TMR was 1.63 Mcal of net energy of lactation (NE_L) and 143 g of crude protein (CP) per kilogram of dry matter (DM) in LP and 1.76 Mcal of NE_L and 183 g of CP per kilogram DM in EL. Blood samples were obtained between 0900 and 1000 h via coccygeal venipuncture throughout the study. For the purpose of the present study, plasma samples came from four cows sampled at fixed times relative to parturition (for sampling times, see Fig. 2 below).

A second study was used to examine the MW distribution of plasma adiponectin in LP and EL and to assess the effect of lactation on WAT expression of ER proteins involved in assembly of adiponectin oligomers. Ten cows were studied in LP on d -28 ± 2 (relative to parturition on d 0) and again in on d 8 of EL as described (26). Composition of the TMR was 1.5 Mcal of NE_L and 140 g of CP per kilogram DM during LP and 1.5 Mcal of NE_L and 180 g of CP per kilogram DM during EL. On each sampling day, individual blood samples were obtained via intrajugular catheters at 2-h intervals between 0800 and 1600 h, and aliquots of each were pooled into a single composite sample for the day. Biopsies of WAT were obtained immediately after the last blood sampling. The estimated net energy balance at each sampling was +12.1 Mcal/d in LP and −15.2 Mcal/d in EL.

A third study was used to examine the relation between plasma adiponectin and adiponectin mRNA abundance and the effect of EL on expression of adiponectin receptor in liver, skeletal muscle, WAT, and mammary gland (24). Eighteen cows were studied either 40 d before expected parturition (LP) or on d 7 of lactation (EL) (n = 9 at each stage). TMR composition was 1.52 Mcal of NE_L and 142 g of CP per kilogram DM in LP and 1.72 Mcal of NE_L and 179 g of CP per kilogram DM in EL. For each cow, individual blood samples were obtained via intrajugular catheters at hourly intervals for 3 h and composited into a single plasma sample representative of the sampling period. Biopsies of liver, WAT, and skeletal muscle were obtained simultaneously on the next day. The net energy balance was not estimated in this experiment, but the plasma concentration of nonesterified fatty acids (NEFA), a metabolite that circulates in proportion to energy insufficiency, was, respectively, 182 and 791 μM in LP and EL cows. Mammary biopsies were also obtained from additional groups of cows (n = 5–7 cows at each stage) 30 d before expected parturition (LP), on the day of parturition (d 0), and on d 21 of lactation (EL) (24).

Gel filtration column chromatography

Plasma samples were mixed 1:1 with elution buffer [25 mM Tris-HCl (pH 7.4) with 100 mM NaCl]. Diluted plasma samples were fractionated on a Protein Pak 300SW (8.0 × 300 mm) column attached to a high-performance liquid chromatography apparatus (Waters 650E, Advanced Protein Purification System; Millipore, Billerica, MA). Separation was performed at a flow rate of 0.75 ml/min with collection of 150-μl fractions. Before and after plasma fractionation, separation was calibrated under identical conditions with pure protein standards ranging from 66 to 669 kDa (Molecular Weight Markers for Liquid Chromatography; Sigma, St. Louis, MO). Fractions encompassing the elution of adiponectin oligomers were analyzed by Western blot analysis (see below).

Production of antibodies against bovine adiponectin and Western blot analysis

A peptide corresponding to the 14 amino-terminal residues of bovine adiponectin (Ad_{N14}) was conjugated to keyhole limpet hemocyanin using sulfhydryl chemistry (Sigma Genosys, Woodlands, TX). Two hundred micrograms of conjugated Ad_{N14} were emulsified with an equal volume of complete Freund's adjuvant and injected into two New Zealand White rabbits. Secondary immunizations were performed on d 14, 28, 42, 56, and 70 (relative to primary immunization) followed by bleeding on d 84.

SDS-PAGE separation was performed under reducing or nonreducing conditions. For reducing conditions, samples (1.5 μl of 1:10 dilution for plasma, 7.5 μl for gel chromatography fractions, and 20 μg of protein lysates from muscle, liver, or WAT) were mixed with equal volumes of 2× Laemmli buffer containing dithiothreitol and mercaptoethanol and boiled for 10 min. They were electrophoresed on 10–13% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience, Keene, NH). The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) [0.05 M Tris (pH 7.4), 0.2 M NaCl, and 0.1% Tween 20] containing 5% wt/vol nonfat dried skim milk (NFM) for 1 h at room temperature and incubated with bovine adiponectin antiserum (1:1000 dilution) in blocking buffer for 2 h at room temperature. For chemiluminescence detection, membranes were incubated with goat antirabbit IgG-horseradish peroxidase (1:5000 dilution) in blocking buffer for 1 h at room temperature, and signals were developed with LumiGLO Western blot chemiluminescence reagent (KPL, Gaithersburg, MD). Alternatively, when performing quantitative analysis, the antirabbit IgG-horseradish peroxidase was replaced with IRDye 800 goat antirabbit secondary antibody (LI-COR Biotechnology, Lincoln, NE). Incubations with the IRDye 800 antibody were performed at 1:20,000 dilution in TBST with 5% NFM for 1 h at room temperature, and signals were quantified with the LI-COR Odyssey infrared imaging system using the 800-nm channel.

For nonreducing conditions, samples (1.5 μl of 1:10 dilution for plasma, 7.5 μl for gel chromatography fractions) were mixed with equal volume of 2× Laemmli buffer devoid of dithiothreitol and mercaptoethanol and left at room temperature for 1 h (27). They were then electrophoresed on 6% SDS-PAGE gels. After blotting, nitrocellulose membranes were blocked in 1× TBST with 5% NFM for 1 h at room temperature. They were incubated in 1× TBST for 2 h with a 1:10,000 dilution of rabbit antibody A6354 (Sigma) detecting adiponectin oligomers separated under nonreducing conditions (27). This antibody recognizes a motif located between amino acids residues 225 and 241 of human adiponectin that is conserved in bovine adiponectin. Detection and quantification were performed using the IRDye 800 goat antirabbit secondary antibody and the LI-COR system as described above.

Gene expression analysis

Total RNA was isolated and purified using RNeasy minicolumns and on-column ribonuclease-free deoxyribonuclease treatment (QIAGEN, Inc., Valencia, CA). Quantity and integrity of RNA was determined using the RNA Nano Lab chip kit and bioanalyzer (Agilent, Palo Alto, CA). Reverse transcriptase reactions were performed with 2 μg of RNA in a 20-μl volume using the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA). Real-time PCR assays were performed in duplicate in a 25 μl volume using Power SYBR Mix (Applied Biosystems). Reactions contained diluted cDNA (25 ng, except 2.5 ng for 18S reactions) and 500 nM of each primer (see Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Primer pairs were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For all genes, each member of the primer pair was located in adjoining exons and shown by blast analysis to anneal exclusively to the target sequence. For all primer pairs, amplification was greater than 0.95 efficient and yielded a single product as shown by melting curve analysis. All mRNA data comparing expression between LP and EL were analyzed using a relative standard curve based on serial 2-fold dilutions of pooled cDNA prepared from WAT samples (28). Unknown sample expression was determined from the standard curve, adjusted for invariant control expression, and reported as a fold difference as indicated in the figure legends. 18S was used as the invariant control because physiological state had no effect on its expression in WAT. Any mRNA with cycle number greater than 34 was declared undetectable. To compare the relative expression of AdipoR1 and AdipoR2 in various tissues, the efficiency-corrected cycle threshold value method was used with all assays performed at the same detection threshold (28, 29). PCR efficiencies (E) were calculated from the slope of the standard curve using $E = 10^{(-1/slope)}$ and the quantity of AdipoR1, AdipoR2, and 18S RNA calculated as quantity $= E^{-Ct}$ (28). An arbitrary expression level was obtained by normalizing the efficiency-corrected value for each receptor to 18S expression (28).

Statistical analysis

Data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute, Raleigh, NC). In the case of the plasma adiponectin profile, data were analyzed by a model accounting for time as the fixed effect and animal as the random effect. The effect of time was partitioned into linear, quadratic, and cubic contrasts. Relative receptor expression data were analyzed by a model accounting for receptor subtype (AdipoR1 or AdipoR2) and tissue (liver, muscle, adipose tissue, and mammary gland) as fixed effects and animal as the random effect. Relative receptor expression was then compared across tissue by pairwise comparisons with Tukey adjustment. For all other experiments, data were analyzed with a mixed model accounting for physiological state (LP *vs.* EL) as a fixed effect and cow as the random effect. The level of statistical significance was set at $P < 0.05$.

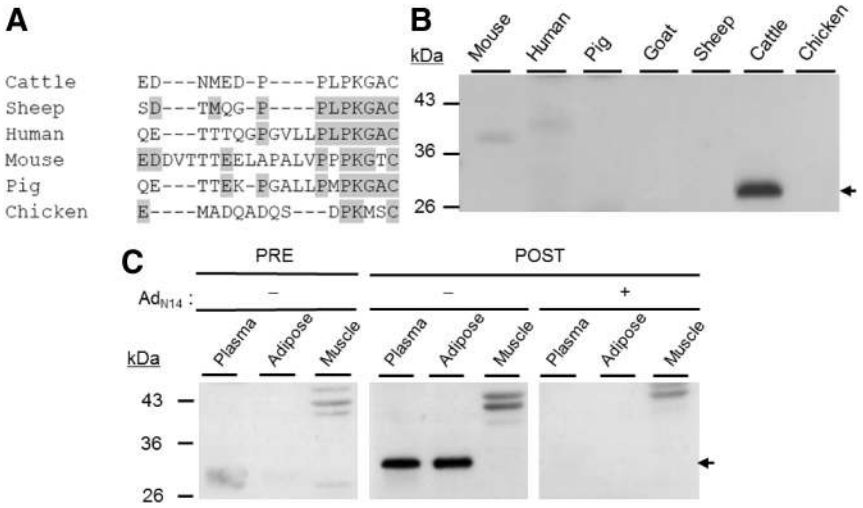

FIG. 1. Development of a bovine adiponectin antiserum. A, The sequence of the bovine peptide used for immunization (Ad_{N14}) is shown at the *top* followed by the corresponding sequence of adiponectin in selected species. *Shading* indicates identity with the corresponding bovine residue. B, Plasma from the indicated species was analyzed by SDS-PAGE under reducing conditions. After electroblotting, the membrane was incubated with the bovine adiponectin antiserum. The *single arrow on the right* indicates the position of the adiponectin signal, whereas positions of MW markers are shown on the *left*. C, Bovine plasma and protein lysates from bovine adipose tissue and skeletal muscle (20 μg) were analyzed by SDS-PAGE under reducing conditions. Immunoblotting was performed with preimmune rabbit serum (PRE) or the bovine adiponectin antiserum (POST) in the absence (–) or presence (+) of 20 μg/ml of Ad_{N14}. The *single arrow on the right* indicates the position of the adiponectin signal, whereas positions of MW markers are shown on the *left*.

Results

Profile of plasma adiponectin during the transition from pregnancy to lactation

We immunized rabbits with a 14-amino acid peptide (Ad_{N14}) corresponding to the hypervariable region of bovine adiponectin and located at the amino-terminal end of the mature protein (Fig. 1A). When used in Western immunoblotting, the antisera detected an approximately 30 kDa protein in bovine plasma and WAT lysates resolved by reducing SDS-PAGE (Fig. 1, B and C). No signal of approximately 30 kDa was detected in plasma of other species or in lysates prepared from bovine muscle. These data suggest that the antiserum recognizes an epitope present in the first seven amino acids of Ad_{N14}, which are highly divergent across species, including in the related sheep (Fig. 1A). The antiserum was specific for adiponectin as shown by elimination of the 30 kDa signal in bovine plasma and WAT lysates when immunoblotting was performed in presence of saturating amounts of Ad_{N14} (Fig. 1C).

The effect of the transition period on plasma adiponectin was examined in a randomly selected group of four cows used in a previous experiment (23). Energy indicators varied in a manner typical of high-yielding dairy cows, with net energy balance and plasma NEFA averaging +6.2 Mcal/d and 123 μM in LP *vs.* −9.1 Mcal/d and 397 μM during the first 3 wk of lactation ($P <$ 0.01 for all, pregnancy *vs.* first 3 wk of lactation). By d 56 of lactation, net energy balance and plasma NEFA were +7.8 Mcal/d and 129 μM and no longer differed from LP values. The profile of plasma adiponectin is shown in Fig. 2. Plasma adiponectin varied in quadratic fashion with highest levels in LP, a maximal reduction of 45% on the day after parturition and a progressive return to LP values by d 56 of lactation ($P <$ 0.01) (Fig. 2B).

Molecular weight distribution of bovine adiponectin during the transition from pregnancy to lactation

In humans and rodents, plasma adiponectin circulates in HMW complexes of more than 540 kDa, MMW complexes of approximately 180 kDa, and LMW complexes of approximately 90 kDa (10–12). To determine the effect of the periparturient period on the distribution of circulating adiponectin, plasma samples were obtained 28 d before parturition and again on d 8 of lactation from an individual cow and fractionated by gel filtration chromatography on a column capable of resolving proteins with apparent MW ranging from 66 to 669 kDa. Fractions spanning the MW range of adiponectin complexes were analyzed by reducing SDS-PAGE and Western immunoblotting (Fig. 3A). Adiponectin eluted as a single peak consistent with the majority of adiponectin circulating in HMW complexes. Analysis of EL plasma yielded a similar fractionation profile except that total adiponectin abundance was reduced. Similar results were obtained in two other cows (Supplemental Fig. 1A).

To confirm these results, plasma and chromatography fractions eluting at the expected mass of the HMW, MMW, and LMW complexes (fractions 19, 27, and 33) were analyzed by nondenaturing SDS-PAGE (27). LP plasma yielded a single adiponectin signal at a size substantially bigger than 250 kDa (Fig. 3B). This signal was prominent in fraction 19 but barely visible in fraction 27; no signal was seen in fraction 33. Similar results were obtained with EL plasma (Fig. 3B) and confirmed in two other animals (Supplemental Fig. 1B). These data suggest that bovine adiponectin circulates predominantly in HMW complexes in LP plasma and that this distribution is unaffected in EL.

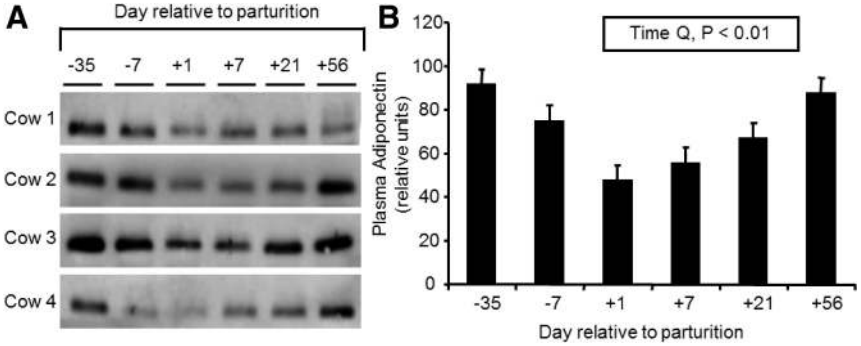

FIG. 2. Profile of plasma adiponectin abundance during the periparturient period. Multiparous dairy cows were studied during the period between d −35 and +56 (relative to parturition on d 0). A, Plasma was obtained at the indicated times and analyzed by SDS-PAGE under reducing conditions. Adiponectin was measured by Western immunoblotting. Fluorescence signals were quantified with the LI-COR Odyssey infrared imaging system. Data are shown for four individual cows. B, *Each bar* represents the mean ± SE of the plasma adiponectin signal (n = 4). The significance level of the quadratic effect of time (Time, Q) is given.

Early lactation reduces adipose tissue expression of genes involved in the formation of adiponectin oligomers

The major source of adiponectin in adult animals is WAT, but expression has also been detected in liver and muscle in chicken (30) and mammary gland in cattle (31). To determine whether mRNA abundance contributes to the reduction of plasma levels in EL, adiponectin expression was measured in biopsies of WAT, liver, and skeletal muscle obtained simultaneously 40 d before parturition in LP and on d 7 of EL. In these cows, plasma adiponectin was decreased by 32% from LP to EL ($P < 0.001$) (Fig. 4A). Adiponectin expression in WAT did not vary between LP and EL (Fig. 4C) even though expression of the WAT-specific hormone leptin was reduced by 75% over this interval ($P < 0.05$) (Fig. 4B). Adiponectin expression was always absent in liver but could be detected in skeletal muscle (Fig. 4C). Skeletal muscle expression, however, was less than 2% of the expression seen in LP WAT and did not change in EL. Adiponectin expression in the mammary gland was examined in a separate group of cows undergoing mammary biopsies during the periparturient period. Adiponectin was detected in three out of seven cows in LP, five out of seven cows on the day of parturition, and four out of five cows in EL and never exceeded 0.05% of LP expression in WAT (data not shown). Therefore, the mammary gland is not a meaningful source of adiponectin, and the reduction in plasma adiponectin in EL is not caused by reduced adiponectin mRNA expression.

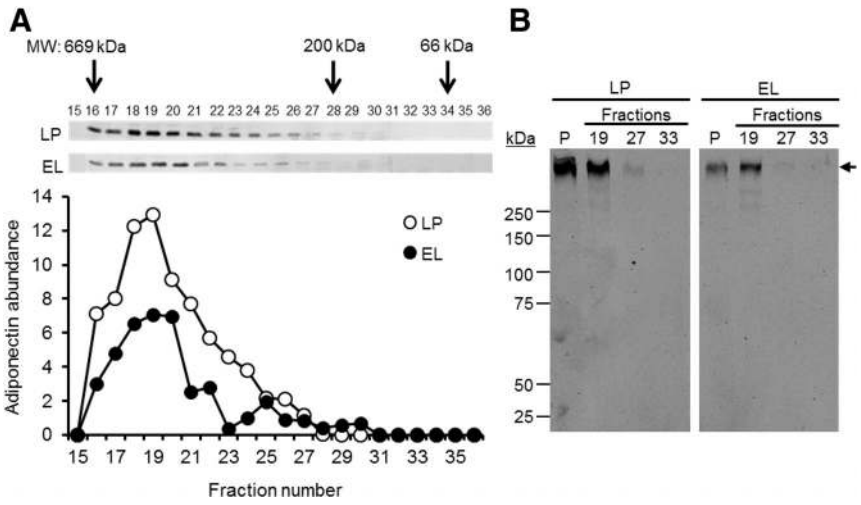

FIG. 3. Molecular weight distribution of plasma adiponectin during the periparturient period. Plasma was obtained from an individual cow in LP (d −32 relative to parturition) and again in EL (d 8 of lactation). A, Plasma samples were fractionated by gel chromatography on a calibrated Protein Pak 300SW column. Fractions were analyzed by SDS-PAGE under reducing conditions, and signals were detected by Western immunoblotting. Fluorescence signals were quantified with the LI-COR Odyssey infrared imaging system. Signal intensity was plotted for each fraction obtained with LP and EL plasma. Positions of the MW markers and bands visualized by Western immunoblotting are shown *above the graph*. B, Plasma (P) and gel chromatography fractions eluting at the approximate molecular size of HMW, MMW, and LMW (fractions: 19, 27, and 33) were analyzed by SDS-PAGE under nonreducing conditions. Adiponectin was measured by Western immunoblotting. Fluorescence signals were quantified with the LI-COR Odyssey infrared imaging system. The *single arrow on the right* indicates the position of the adiponectin signal, whereas positions of MW markers are shown on the *left*.

The assembly of adiponectin into HMW complexes involves a subset of ER-associated proteins (11, 32). To study the effects of EL on the expression of the corresponding genes, WAT biopsies were obtained from another group of cows in LP and again in EL. In this experiment, plasma adiponectin was reduced by 40% between LP and EL ($P < 0.01$) (Fig. 5A), again in the absence of any change in WAT adiponectin mRNA (Fig. 5B). The reduction in plasma adiponectin was associated with reduced expression of the ER chaperone glucose-regulated protein, 78 kDa (Grp78), the thiol-retaining protein ER protein 44 (Erp44), and the protein disulfide isomerase family A, member 6 (Pdia6) ($P < 0.05$ or less) (Fig. 5C) but had no effect on ERO1-like protein α (Ero1-Lα), disulfide-bond A oxidoreductase-like protein (DsbA-L), and Golgi-localizing γ adaptin ear homology domain ARF-binding protein (GGA1) expression. These data raise the possibility that formation of

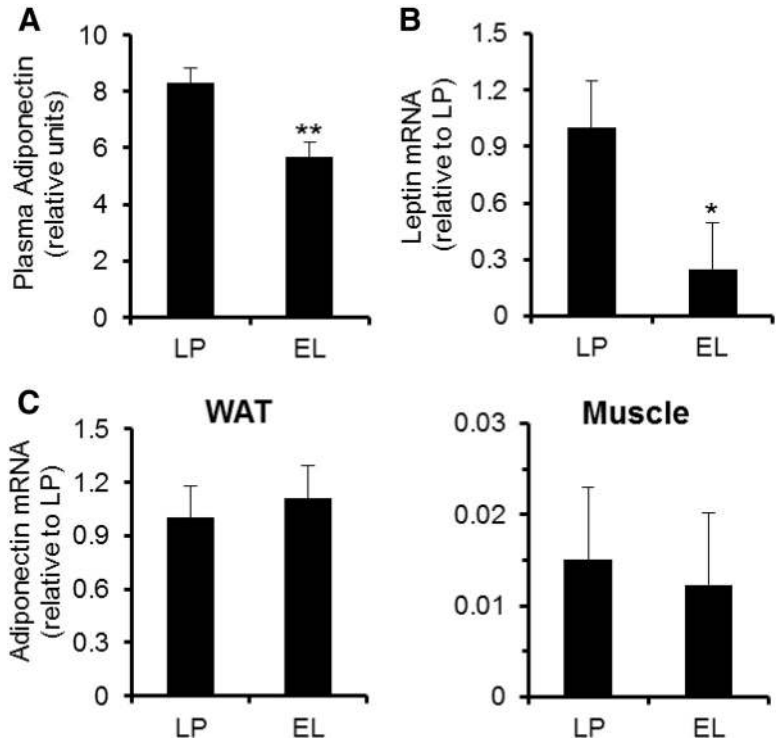

FIG. 4. Effect of early lactation on plasma adiponectin and adiponectin mRNA expression. Plasma and tissues were obtained from multiparous dairy cows studied 40 d before expected parturition (LP) and on d 7 of lactation (EL). A, Plasma was analyzed by SDS-PAGE under reducing conditions, and adiponectin was measured by Western immunoblotting. Fluorescence signals were quantified with the LI-COR Odyssey infrared imaging system. *Each bar* represents the mean ± SE of the adiponectin signal (n = 9). **, $P < 0.001$. B, Leptin mRNA expression was measured in WAT by quantitative real-time PCR. Expression is relative to LP WAT. *Each bar* represents the mean ± SE of leptin mRNA (n = 9). *, $P < 0.05$. C, Adiponectin mRNA expression was measured in WAT and skeletal muscle of each animal by quantitative real-time PCR. Expression in both tissues is relative to LP WAT. *Each bar* represents the mean ± SE of adiponectin mRNA (n = 9).

HMW adiponectin is impaired in EL and contributes to the reduction of plasma adiponectin.

Early lactation increases AdipoR1 expression in muscle and AdipoR2 expression in liver

Previous studies in cattle have measured the effect of EL on AdipoR1 and AdipoR2 expression in WAT (33) but have not considered the two other major insulin-responsive tissues (*e.g.* muscle and liver) and the mammary gland. First, we compared the transcript abundance of each receptor across these tissues in LP cows. AdipoR1 expression in muscle was 6.6-fold higher than in liver and 2-fold higher than in WAT and mammary gland ($P < 0.05$ or less) (Fig. 6A). AdipoR2 expression was 2.5- to 5.5-fold higher in WAT than in the other surveyed tissues ($P < 0.01$ or less).

When transcripts of each receptor were compared within tissue, AdipoR1 abundance was approximately 2-fold higher than AdipoR2 in muscle and mammary gland ($P < 0.01$ or less) (Fig. 6A). The reciprocal situation occurred in liver and WAT, whereby AdipoR2 was 3- to 4-fold more abundant than AdipoR1 ($P < 0.01$ or less).

Finally, we measured changes in AdipoR expression from LP to EL. EL caused a 2.5-fold increase in AdipoR1 expression in muscle ($P < 0.05$) (Fig. 6B) but had no effect on its expression in liver or WAT. In contrast, EL increased AdipoR2 expression 2.5-fold in liver ($P < 0.05$) but had no effect in other tissues. The transition from LP to EL had no effect on the expression of either receptor in the mammary gland (Fig. 6C). These data suggest that liver and muscle respond to the adiponectin deficit of EL by up-regulating expression of their dominant AdipoR subtype.

Discussion

Insulin resistance persists after parturition in high-producing dairy cows in contrast to the reversal seen in rodents and primates (3, 4, 6, 7). This is likely a consequence of the greater precarious nature of the glucose economy in EL than in LP in these animals. This situation is the product of two factors. First, dairy cows have inadequate voluntary feed intake in EL and thus insufficient substrates of dietary origin to fulfill the glucose demand of the mammary gland for lactose synthesis (6, 8). Second, the fraction of available glucose devoted to milk synthesis reaches 85% in EL in highly productive dairy cows, compared with approximately 50% for the products of conception in LP (8). Persistence of insulin resistance after parturition creates a metabolic milieu favoring mammary glucose uptake, because like in the placenta, this process occurs in an insulin-independent manner (1). The mechanisms preserving insulin resistance in EL dairy cows are not well understood, prompting us to examine whether the potent insulin-sensitizing hormone adiponectin could be involved.

To our knowledge, regulation of plasma adiponectin has not been examined in cattle, reflecting perhaps lack of suitable antibodies. Using a bovine-specific antibody, we show that plasma adiponectin drops rapidly during the last week of pregnancy, reaches a nadir at parturition, and rebounds over the next 7 wk of lactation. In lactating

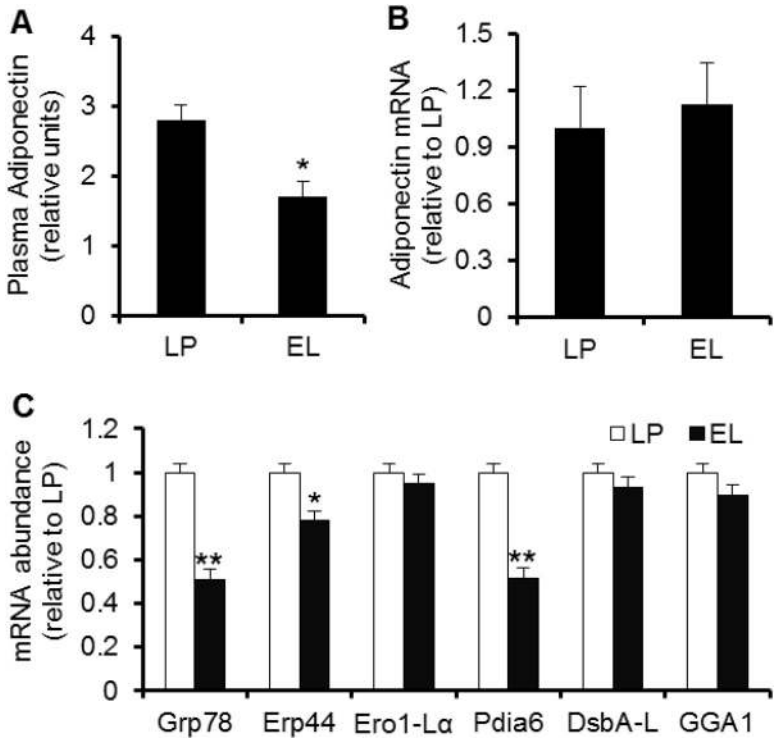

FIG. 5. Effect of early lactation on adipose tissue expression of ER-associated proteins involved in the formation of adiponectin complexes. Adipose tissue was obtained from multiparous dairy cows 28 d before expected parturition (LP) and on d 8 of lactation (EL). A, Plasma was analyzed by SDS-PAGE under reducing conditions, and adiponectin was measured by Western immunoblotting. Fluorescence signals were quantified with the LI-COR Odyssey infrared imaging system. *, $P < 0.01$. B, Adiponectin mRNA expression was measured in WAT by quantitative real-time PCR. Expression is relative to LP WAT. *Each bar* represents the mean ± SE of adiponectin mRNA (n = 10). C, mRNA expression of ER-associated proteins was measured in WAT by quantitative real-time PCR. Expression of each gene is given relative to LP level. For each indicated gene, *bars* represent the mean ± SE of the corresponding mRNA expression (n = 10). Genes are glucose-regulated protein 78 kDa (Grp78), the thiol-retaining protein ER protein 44 (Erp44), ERO1-like protein α (Ero1-Lα), disulfide-bond A oxidoreductase-like protein (DsbA-L), and Golgi-localizing γ adaptin ear homology domain ARF-binding protein (GGA1). *, $P < 0.05$; **, $P < 0.0001$.

women, plasma adiponectin did not differ when measured during the third trimester of pregnancy and on d 3 or 30 of lactation (34). Most other studies made no comments on the lactation status of the women studied and observed comparable plasma adiponectin in LP and after parturition (35–37). In contrast, mice had lower plasma adiponectin after 1 wk of lactation than during the third week of gestation (38). The reduction in plasma adiponectin in EL dairy cows and mice could relate to the greater energetic intensity of their lactation and, therefore, a greater need to favor mammary glucose uptake.

Another important variable to consider in the context of insulin action is the oligomeric nature of plasma adiponectin. This is because HMW adiponectin correlates more strongly with insulin action than total adiponectin, both in conditions of insulin resistance (*e.g.* obesity and type 2 diabetes) and after its reversal by weight loss or treatment with PPARγ agonists (12, 13, 20, 21, 39, 40). The MW distribution of plasma adiponectin varies across species. In healthy women, HMW, MMW, and LMW complexes account, respectively, for 55, 28, and 17% of total adiponectin (41). In female mice, plasma adiponectin is found nearly exclusively in a 2:1 ratio of HMW and MMW complexes, whereas it is found exclusively in HMW complexes in chicken (41, 42). We are not aware that this issue has been examined in cattle other than during purification from fetal calf serum; these efforts suggested that most, if not all, adiponectin occurs in a HMW complex containing 18 monomers during fetal life (43, 44). To examine the MW distribution of plasma adiponectin in adult cattle, we adopted the procedure of Scherer and coworkers (41), whereby plasma is fractionated by gel filtration chromatography followed by SDS-PAGE analysis of fractions under reducing conditions. We also analyzed plasma and fractions under conditions preserving the oligomeric structure (*i.e.* nonheating and nonreducing conditions) (27). Both methods lead to the same conclusions, namely that most bovine adiponectin circulates in HMW complexes in LP and that this distribution remains unaffected in EL. Accordingly, our studies rule out a shift in the MW distribution of adiponectin as a factor involved in the preservation of insulin resistance in periparturient dairy cattle.

Next, we sought to determine whether changes in WAT mRNA accounted for reduced plasma adiponectin in EL. In two independent experiments, we observed invariant WAT mRNA levels through the periparturient period, in agreement with a recent study performed in high-yielding dairy cows (33). These results, however, are contrary to findings of lower WAT adiponectin mRNA in peak-lactating than nonlactating cows in an earlier study (45). The reason for this discrepancy is unclear, but we note that the pregnancy status of the nonlactating group was not described in that study and that WAT was sampled outside the periparturient period in both peak-lactating and nonlactating groups. Invariant adiponectin mRNA during this period is not unique to dairy cows, because WAT expression holds steady through pregnancy and lactation in rats (46, 47). Nor is it unique to lactation, because steady WAT

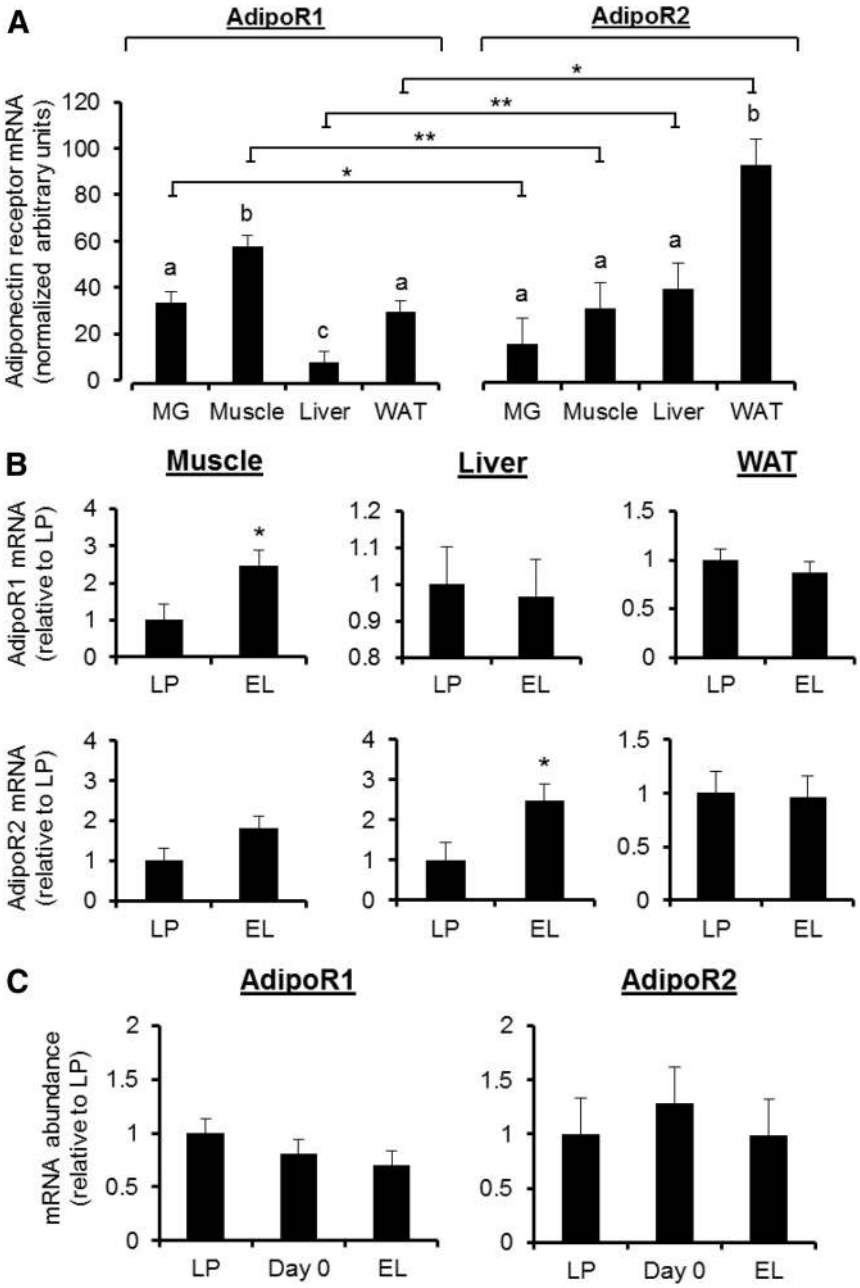

FIG. 6. Effect of early lactation on expression of adiponectin receptors. A, Expression of AdipoR1 and AdipoR2 was measured by quantitative real-time PCR in multiparous Holstein cows studied 40 d before expected parturition (LP). Analysis compared the expression of each receptor across mammary gland (MG), muscle, WAT, and liver (a–c, $P < 0.05$) or expression of both receptors within tissues (*, $P < 0.01$; **, $P < 0.001$). *Each bar* represents the mean ± SE of receptor expression (n = 9). B, Expression of AdipoR1 and AdipoR2 was measured by quantitative real-time PCR in multiparous Holstein cows studied 40 d before expected parturition (LP) and on d 7 of lactation (EL). For each tissue and receptor, expression is relative to LP and represents the mean ± SE (n = 9). *, $P < 0.05$. C, AdipoR1 and AdipoR2 mRNA expression was measured by quantitative real-time PCR in the mammary gland of multiparous cows at 30 d before expected parturition (LP), on the day of parturition (d 0), and on d 21 of lactation (EL). For each receptor and time, expression is relative to LP mammary gland and represents the mean ± SE (n = 5–7).

mRNA expression has been observed frequently with other treatments causing changes in plasma adiponectin (38, 48, 49). We also measured adiponectin mRNA in the mammary gland where expression has been previously reported (31). Our quantitative data, however, show negligible production by the mammary gland in either LP or EL and likely reflects contamination of secretory tissue by intramammary WAT depots. This inference is supported by detection of low expression in muscle, another tissue known to harbor small WAT depots.

Recent studies have shown that the assembly of adiponectin into oligomeric complexes impacts adiponectin production (50–53). Adiponectin assembly occurs in the ER and is coordinated by residing chaperones, foldases, and oxidoreductases (11, 32). The first step is trimer formation, a process driven by hydrophobic interactions between adiponectin monomers and assisted by the chaperone Grp78 (32). This is followed by thiol-mediated retention of the emerging complex by the integral ER protein Erp44 (51). This retention allows the oxido-reductase Ero1-Lα to transfer disulfide bonds to adiponectin via protein disulfide isomerase action and, when fully formed, to release HMW adiponectin from Erp44 (50, 51). DsbA-L and GGA1 are other proteins, respectively, involved in formation and transport of HMW adiponectin (52, 54). Reduced plasma adiponectin and insulin action in obesity are associated with lower Erp44, Ero1-Lα, and DsbA-L in WAT, whereas the opposite is seen when both variables are normalized by PPARγ agonist treatment (51, 52). Consistent with reduced capacity of WAT to assemble adiponectin in HMW complex after parturition, Grp78, Erp44, and Pdia6 expression was lower in EL than in LP. The functional significance of these changes is supported by reduced production of total and HMW adiponectin in the absence of altered adiponectin mRNA when individual members of this ER system are knockdown *in vitro* (50–52).

In both mice and humans, the insulin resistance of obesity and type 2 diabetes is associated with changes in adi-

ponectin receptor expression. Adiponectin signals via two related receptors known as AdipoR1 and AdipoR2 (10, 22). Our first effort was to measure the relative expression of both receptors in the three major insulin-responsive tissues of LP cows. In agreement with surveys in human and mice (22), we find that AdipoR1 is higher than AdipoR2 in muscle, whereas AdipoR2 dominates in liver. Unlike the mouse, however, the site of highest AdipoR2 expression in our work was not liver but WAT, as recently shown in chicken and pigs (55, 56). Our most important finding, however, is the ability of EL to stimulate expression of the predominant receptor subtype in two insulin-sensitive tissues, namely AdipoR1 in muscle and AdipoR2 in liver. This induction may be significant in EL when the rate of lipid use by liver and skeletal muscle increases substantially (1, 6). This is because AdipoR1 promotes lipid use in muscle via a pathway involving AMP-activated kinase (AMPK) and its positive effects on mitochondrial function (57), whereas AdipoR2 does so in liver via the master regulator of oxidative metabolism, PPARα (22). Accordingly, receptor induction may be a mechanism facilitating lipid oxidative capacity of muscle and liver in EL in the face of decreasing plasma adiponectin. In contrast, no changes were detected in the expression of either receptor in the mammary gland or WAT. A lack of regulation of AdipoR expression in these tissues is not surprising: the mammary gland takes up glucose independently of insulin and performs negligible fatty acid oxidation (1, 6), whereas WAT is nearly devoid of biosynthetic activity in EL (6, 8), abrogating the need for glucose uptake or lipid oxidation. It will be important in future work to assess the functional consequences of changes in plasma adiponectin and receptor expression in tissues on functional targets such as AMPK.

In summary, we show that the insulin-sensitizing hormone adiponectin is reduced around parturition in the dairy cow. This reduction occurred without any change in the MW distribution of plasma adiponectin or adiponectin mRNA abundance in WAT but involved repression of a subset of proteins implicated in the formation of HMW adiponectin. These changes overlap with the maintenance of insulin resistance in EL, a physiological adaptation that favors the partitioning of available glucose to the mammary gland and is favorable to most individuals. If too pronounced, however, insulin resistance leads to excessive lipid mobilization from WAT in EL and to the development of metabolic diseases such as fatty liver and ketosis (58). Future studies need to address the possibility that an excessive reduction in plasma adiponectin around parturition contributes to maladaptive insulin resistance seen in the subset of dairy cows afflicted by metabolic diseases.

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