Adipose tissue remodeling in late-lactation dairy cows during feed-restriction-induced negative energy balance

G. Andres Contreras,^{*1} Kyan Thelen,^{*} Sarah E. Schmidt,[†] Clarissa Strieder-Barboza,^{*} Courtney L. Preseault,[‡] William Raphael,^{*} Matti Kiupel,[‡] John Caron,^{*} and Adam L. Lock[†]

^{*}Department of Large Animal Clinical Sciences,

[†]Department of Animal Science, and

[‡]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing 48824

ABSTRACT

Excessive rates of demand lipolysis in the adipose tissue (AT) during periods of negative energy balance (NEB) are associated with increased susceptibility to disease and limited lactation performance. Lipolysis induces a remodeling process within AT that is characterized by an inflammatory response, cellular proliferation, and changes in the extracellular matrix (ECMT). The adipose tissue macrophage (ATM) is a key component of the inflammatory response. Infiltration of ATM-forming cellular aggregates was demonstrated in transition cows, suggesting that ATM trafficking and phenotype changes may be associated with disease. However, it is currently unknown if ATM infiltration occurs in dairy cows only during NEB states related to the transition period or also during NEB-induced lipolysis at other stages of lactation. The objective of this study was to evaluate changes in ATM trafficking and inflammatory phenotypes, and the expression of genetic markers of AT remodeling in healthy late-lactation cows during feed restriction-induced NEB. After a 14-d (d −14 to d −1) preliminary period, Holstein cows were randomly assigned to 1 of 2 feeding protocols, ad libitum (AL) or feed restriction (FR), for 4 d (d 1–4). Caloric intake was reduced in FR to achieve a targeted energy balance of −15 Mcal/d of net energy for lactation. Omental and subcutaneous AT samples were collected laparoscopically to harvest stromal vascular fraction (SVF) cells on d −3 and 4. The FR induced a NEB of -14.1 ± 0.62 Mcal/d of net energy for lactation, whereas AL cows remained in positive energy balance (3.2 ± 0.66 Mcal/d of NE_L). The FR triggered a lipolytic response reflected in increased plasma nonesterified fatty acids (0.65 ± 0.05 mEq/L on d 4), enhanced phosphorylation of hormone sensitive lipase, and reduced adipocyte diameter.

Flow cytometry and immunohistochemistry analysis revealed that on d 4, FR cows had increased numbers of CD172a⁺, an ATM (M1 and M2) surface marker, cells in SVF that were localized in aggregates. However, FR did not alter the number of SVF cells expressing M1 markers (CD14 and CD11c) or M2 markers (CD11b and CD163). This finding contrasts with the predominately M1 phenotype observed previously in ATM from clinically diseased cows. No changes were observed in the expression of ECMT-related or cell proliferation markers. In summary, an acute 4-d lipolytic stimulus in late-lactation dairy cows led to ATM infiltration with minimal changes in inflammatory phenotype and no changes in ECMT. These results underscore that physiological changes related to parturition, the onset of lactation, extended periods of lipolysis, or a combination of these can induce intense AT remodeling with enhanced ATM inflammatory phenotype expression that may impair the metabolic function of AT in transition dairy cattle.

Key words: adipose tissue remodeling, fatty acid, lipolysis, macrophages

INTRODUCTION

Adipose tissue (AT) lipolysis is a dynamic physiological response that involves not only the release of free fatty acids from adipocyte triglyceride reserves but also a remodeling process (Kosteli et al., 2010). The remodeling is accompanied by an inflammatory response with immune cell migration, proliferation of cellular components of the stromal vascular fraction (SVF), and changes in the extracellular matrix (ECMT) within AT (Rutkowski et al., 2015). An increased rate of total lipolysis, defined as the sum of basal and demand lipolysis, is the triggering stimulus for AT remodeling (Kosteli et al., 2010). The rate of basal lipolysis is determined by adipocyte TG content (Arner and Langin, 2014). In contrast, demand lipolysis is regulated hormonally in response to energy requirements. In dairy

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¹Corresponding author: contre28@msu.edu

cattle, pronounced and protracted demand lipolysis in periods of negative energy balance (**NEB**), such as during the transition period, are associated with increased susceptibility to disease (Ospina et al., 2013). Despite this important epidemiological association, features of AT remodeling during lipolysis remain poorly understood. Although immune cell migration and activation are important components of the AT remodeling process in nonruminant species (Ferrante, 2013), little is known about these processes in ruminants. We recently demonstrated macrophage infiltration into AT of transition dairy cows with displaced abomasum (**DA**) and intense demand lipolysis (Contreras et al., 2015). In those transition cows, adipose tissue macrophages (**ATM**) infiltrated omental (**OM**) and subcutaneous (**SC**) fat, forming cellular aggregates similar to those seen in obese rodents and humans (Contreras et al., 2015). In healthy postpartum heifers, Akter et al. (2012) reported increased numbers of ATM in OM and mesenteric AT depots 1 d after parturition. Collectively, these findings suggest ATM trafficking changes occur in the bovine periparturient period and may be associated with the pathogenesis of metabolic diseases; however, it is currently unknown if ATM infiltration occurs in dairy cattle only during NEB states related to the transition period or also during NEB-induced demand lipolysis at other stages of lactation.

The macrophage is the most abundant immune cell type in the SVF of AT in domestic cattle, sheep, goats, cats, and dogs (Ampem et al., 2016). In non-pregnant, nonlactating cows, this mononuclear cell type represents around 10% of the SVF population (Contreras et al., 2015). During intense lipolytic events, ATM are the key component of the active inflammatory response and play a major role in the remodeling process of AT in rodents and humans (Kosteli et al., 2010; Suganami and Ogawa, 2010). The ATM remove fatty acids that are released in excess during lipolysis and reduce lipotoxicity in interstitial tissue. The ATM are also involved in the recruitment of new adipocyte progenitors by secreting chemotactic proteins such as osteopontin (Lee et al., 2013). The specific inflammatory phenotype of ATM is dependent on the types and concentrations of local cytokines, chemokines, and fatty acids that shape gene and protein expression profile during inflammation. Classical phenotype ATM (M1) stimulate pro-inflammatory pathways and secrete cytokines including tumor necrosis factor α (**TNF α**) and IL-6. Alternative phenotype ATM (M2) promote inflammation resolution by secreting cytokines such as IL-10 (Ferrante, 2013). Although it appears that M1 ATM are associated with intense periparturient lipolysis (Contreras et al., 2015), it is currently unknown if

lipolysis during feed-restriction-induced NEB in mid and late lactation alters macrophage polarization and therefore could serve as a model for AT remodeling in transition cows.

Besides ATM infiltration, the AT remodeling process is also associated with changes in ECMT in monogastric animals. Increased deposition of collagen VI and thrombospondin during inflammatory responses in AT impairs adipocyte metabolic function by limiting lipid accumulation and insulin signaling (Varma et al., 2008; Pasarica et al., 2009). Less is known about the expression of ECMT proteins in dairy cows during lipolytic responses and their effect on AT metabolic function. In humans and rodents, AT remodeling with excessive infiltration of M1 ATM and increased deposition of ECMT impair adipocyte response to insulin, leading to enhanced total lipolysis that results in chronic elevation of circulating fatty acids. Characterizing AT remodeling in cows during NEB may identify mechanisms that lead to excessive rates of lipolysis that predispose transition cows to disease and poor lactation performance. Therefore, our objective was to evaluate changes in ATM trafficking and inflammatory phenotypes, and the expression of genetic markers of AT remodeling in healthy late-lactation cows during feed restriction-induced NEB.

MATERIALS AND METHODS

Cows

All animal procedures were approved by the Michigan State University Animal Care and Use Committee. Late-lactation ($223 \text{ DIM} \pm 103 \text{ SD}$) bovine leukemia virus seronegative Holstein cows from the Michigan State University Dairy Field Laboratory (East Lansing, MI) were randomly assigned to 1 of 2 feeding protocols: ad libitum (**AL**) or feed-restricted (**FR**) in 2 separate blocks. In block 1, which was performed in August 2014, 8 cows in their second ($n = 4$), third ($n = 2$), and fourth ($n = 2$) lactations were selected. Cows were fed a common diet during a 14-d preliminary period (d -14 to -1) and then randomly assigned based on lactation number to AL ($n = 4$) or FR ($n = 4$) treatments. Average milk yields during the preliminary period were $35.6 \pm 8.8 \text{ kg}$ and $39.4 \pm 6.2 \text{ kg}$ for the AL and FR groups, respectively. The BCS scores before FR were $AL = 3.1 \pm 0.1$ and $FR = 3.3 \pm 0.2$. One cow from AL was removed from the study due to a health event before the beginning of the treatments. Caloric intake was reduced in FR cows for 4 d (d 1 to 4) to achieve a targeted NEB of -15 Mcal/d of NE_L similar to the transition period (3 wk before to 3 wk after calving;

Koltes and Spurlock, 2011). In block 2, which was performed in November 2014, 6 cows in their first ($n = 2$) or second lactation ($n = 4$) were randomly assigned based on lactation number to FR and AL treatments as described for block 1. Two cows from block 1 were used in block 2 but were assigned to the opposite treatment. Preliminary period milk yields for cows in block 2 were 32.6 ± 5.2 (AL) and 29.1 ± 1.4 (FR). The BCS at the end of the preliminary period were 3.6 ± 0.2 and 3.3 ± 0.1 for cows in the AL and FR groups, respectively.

Feed Restriction Protocol

The ingredient and nutrient composition of the diets fed as a TMR in blocks 1 and 2 are described in Table 1. Minerals and vitamins were formulated according to NRC (2001) recommendations. Samples of all diet ingredients (0.5 kg) were collected daily and composited during the preliminary period and FR protocols from d 1 to 4. Samples were analyzed for NDF, CP, starch, and fatty acid concentrations as described by Boerman et al. (2015). Baseline BW, DMI, and the yield of milk and milk components were measured for 5 d before feed restriction. Feed was restricted for 4 d with the quantity of TMR provided to each cow adjusted on d 3 of the feed restriction period to account for changes in milk production. Cows were fed 2 times daily throughout the preliminary and FR periods. Net energy balance was estimated as the difference between NE_L consumed

and used for milk production and maintenance (NRC, 2001).

Sample Collection and Analysis

Blood and milk were collected on d -7 , -3 , -2 , and -1 and then daily from d 1 to 4. Total plasma non-esterified fatty acid (NEFA) concentrations were quantified using the NEFA-HR-2 kit (Wako, Richmond, VA) as described previously (Contreras et al., 2010). Plasma insulin was determined by ELISA following manufacturer guidelines (Bovine Insulin ELISA; Mercodia AB, Uppsala, Sweden) and using a Synergy H1 Microplate Reader (Biotek, Winooski, VT). Intra- and interassay CV were 4.1 and 4.3%, respectively. Circulating glucose values were determined using an Olympus AU640e chemistry analyzer (Olympus America, Center Valley, PA) at the Diagnostic Center for Population and Animal Health (Lansing, MI). Milk samples were analyzed by the Northstar Cooperative Dairy Herd Improvement (Northstar Laboratories, East Lansing, MI) as described previously (Boerman et al., 2015).

Omental and SC AT samples were collected on d -3 and 4 laparoscopically. In brief, cows received local flank anesthesia using an inverted L block with 20 mL of 2% lidocaine hydrochloride (Aspen Veterinary Resources, Greeley, CO). Two 1-cm incisions were made through the skin in the right paralumbar fossa. The external abdominal oblique, internal abdominal oblique, and transverse abdominus muscles were incised. Next, the peritoneum was incised and the laparoscopic equipment was introduced in the incisions, pneumoperitoneum (10 mmHg) was established, and suitable location(s) for OM adipose sampling identified. Using cup biopsy forceps, 3 g of OM tissue were collected. A 3-g SC sample was collected from the area of the incisions before its closure using synthetic absorbable material in a buried suture pattern. Total surgical time was less than 20 min per animal. For cows in block 2, instead of laparoscopic collection of AT on d 4, samples (matching sites used for laparoscopic OM biopsy) were collected postmortem at the Michigan State University Meat Laboratory.

Adipose Tissue Processing

Immediately after harvesting, AT were divided and stored for further processing. Samples for protein analysis were snap frozen and stored at -80°C . For gene expression analysis, samples were stored in RNA Later (Life Technologies, Carlsbad, CA). Samples for histology were immersed in 4% paraformaldehyde (Electron Microscopy Sciences, Hartfield, PA) or 30% sucrose in distilled water (vol:vol). For tissue

Table 1. Ingredient and nutrient composition of the treatment diet

Item	Block 1	Block 2
Ingredient (% of DM)		
Corn silage	21.9	32.1
Alfalfa silage	13.5	16.3
Wheat straw	2.5	—
Dry ground corn	19.6	25.1
High moisture corn	9.1	—
Soybean meal	16.0	15.9
Soy hulls	6.9	—
Cottonseed with lint	7.1	7.2
Vitamin-mineral mix ¹	2.0	2.0
Limestone	0.64	0.66
Sodium bicarbonate	0.73	0.75
Nutrient composition (% of DM)		
DM	56.4	51.6
NDF	30.2	28.0
CP	17.4	17.5
Starch	22.2	29.5
Fatty acid	3.4	3.5

¹Vitamin and mineral mix contained 34.1% dry ground shell corn, 25.6% white salt, 21.8% calcium carbonate, 9.1% Biofos (The Mosaic Co., Plymouth, MN), 3.9% magnesium oxide, 2% soybean oil, and <1% of trace minerals including (g/100 g) 2.0 copper, 12.0 sulfur, 10.5 zinc, 0.3 manganese, 0.25 cobalt, 0.19 iodine, a selenium blend containing 0.006% sodium selenite, and vitamin ADE mixture containing (g/kg) 10.8 retinyl acetate, 0.18 cholecalciferol, and 0.047 DL- α -tocopherol.

fractionation, samples were collected in Krebs Ringer bicarbonate buffered solution containing 50 µg/mL of gentamicin (MP Biochemicals, Solon, OH) and HEPES 10 mM (pH = 7.4) and transported immediately to the laboratory where adipocytes and SVF were obtained by collagenase digestion of AT, as previously described (Contreras et al., 2015).

Flow Cytometry Analysis

Macrophage distribution and phenotype in SVF of AT were determined using flow cytometry as previously described (Contreras et al., 2015). Mouse anti-bovine antibodies were obtained from the Washington State University Monoclonal Antibody Center (Pullman, WA): B cells (S-BAQ44A; 1:100), CD3 (MM1A; 1:100), CD11b (BAQ150A; 1:100), CD11c (BAQ153A; 1:100), CD14 (MM61A; 1:50), CD172a (DH59B; 1:100); and AbD Serotec (Raleigh, NC): CD163 (MCA2311GA; 1:100). Secondary goat anti-mouse FITC was from Jackson ImmunoResearch (West Grove, PA). Flow cytometry analysis was performed using the FACSdiva software on an LSRII Flow Cytometer (BD Biosciences, San Jose, CA).

Quantitative PCR Analysis

Adipose tissue RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's directions and then purified using the RNA Easy Mini Kit (Qiagen, Valencia, CA) as described previously (Contreras et al., 2014). Purity, concentration, and integrity of mRNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) system. All samples had a 260:280 nm ratio between 1.9 and 2.1 and an RNA integrity number >6. Conversion to cDNA was performed using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). All quantitative PCR assays were conducted with TaqMan gene expression primers and reagents from Applied Biosystems. Taq primers were either commercially available or designed from bovine sequences with the Applied Biosystems Pipeline software and synthesized by Applied Biosystems Inc. (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2016-11552>). Samples were assayed in triplicate on the Wafergen Smartchip System (WaferGen Biosystems, Fremont, CA) using cDNA at a primer-efficient concentration of 714 pg/µL. A non-reverse-transcriptase control sample validated absence of genomic DNA. Data are presented as fold changes relative to the AL SC samples. These were calculated from least squares mean differ-

ences according to the formula $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{\text{Target gene}} - Ct_{\text{Control gene}}$, and $\Delta\Delta Ct = \Delta Ct_{\text{Target Sample}} - \Delta Ct_{\text{Calibrator Sample (AL SC)}}$. Appropriate control genes were selected as previously described (Steibel et al., 2009) using Data Assist software (Applied Biosystems) and included *EIF3K*, *B2M*, and *RPS9*. For reporting, expression data were normalized to the mean of these housekeeping genes. All statistical analysis was performed on the ΔCt values as described by Steibel et al. (2009).

Immunohistochemistry

Localization of ATM was determined using immunolabelling of paraffin sections using the Autostainer Link 48 (Dako North America Inc., Carpinteria, CA) automated stainer and a mouse monoclonal anti-CD172a (DH59B; 1:100) antibody (Contreras et al., 2015). The ATM aggregates, defined as clusters of CD172a⁺ cells, were quantified within 100 contiguous adipocytes from 5 randomly selected fields per section. Imaging was performed using an Olympus BX-40 microscope (Olympus, Center Valley, PA). Adipocyte cell diameters were determined as described earlier (Verdes et al., 2013) in hematoxylin- and eosin-stained sections of paraformaldehyde fixed paraffin-embedded tissue. Diameters of 100 adjacent cells from each of 5 randomly selected fields were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Western Blotting

Protein quantification was performed by Western blot analysis as previously described (Contreras et al., 2014). Protein was extracted in RIPA buffer (Teknova, Hollister, CA) containing protease (Roche, San Francisco, CA) and phosphatase (Thermo Scientific, Waltham, MA) inhibitors. Equal sample amounts of protein (30 µg) were electrophoresed on a 4 to 20% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline (TBS) with 0.01% Tween-20 and 5% BSA and washed 3 times with TBS. Membranes were incubated overnight with primary antibodies in TBS with 0.01% Tween-20 and 5% BSA. Antibodies were from Cell Signaling Technology (Danvers, MA) and included rabbit anti-mouse hormone sensitive lipase (HSL, 1:1000, #4107), rabbit anti-mouse phosphorylated HSL serine 660 (1:1,000, #4126), and rabbit anti-human α/β tubulin as loading control (1:1,000, #2148). Following 3 washes with TBS, membranes were incubated with the correspondent anti-host IgG secondary antibody labeled with horseradish peroxidase (1:10,000 dilution in 5% BSA, Jackson ImmunoResearch) for 10 min at room

temperature, washed 3 times with TBS, exposed to horseradish peroxidase substrate (Millipore, Billerica, MA). Protein bands were visualized by chemiluminescence using the ChemiDoc XRS (Bio-Rad, Hercules, CA). Density of the bands was quantified by the Quantity One software (Bio-Rad).

Statistical Analyses

Statistical analysis was conducted using Proc MIXED (SAS version 9.4, SAS Institute Inc., Cary, NC). The production, plasma glucose, and serum insulin data were analyzed with linear models that included the fixed effects of treatment, block, preliminary milk yield (pMilk), and their interactions. pMilk was defined as the milk yield of each cow on d -1 relative to the feed restriction period. The qPCR, Western blot, flow cytometry, immunohistochemistry, and adipocyte diameter data were analyzed with linear models that included the fixed effects of treatment, block, tissue site, and their interactions. Plasma NEFA concentrations were analyzed with a repeated measures model that included the effects of treatment, block, day (repeated), pMilk, and their interactions. An autoregressive covariance structure was selected for the repeated measures model based on the model Akaike information criterion value. Interaction terms were removed from the models when $P > 0.20$. Least squares means differences were determined by t -tests using the PDIF option. Data, with the exception of the gene expression fold changes, are reported as least squares means $\pm$ standard error of the mean. The 95% confidence intervals of the gene expression fold changes (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2016-11552>) were transformed from the 95% confidence intervals of the $\Delta\Delta Ct$ values estimated as least squares mean differences of ΔCt values. Main effects, least squares mean differences, and interaction terms were considered significant when $P \leq 0.05$ and tendencies when $P < 0.10$.

RESULTS

FR Induces NEB and Lipolysis

Whereas AL cows remained in positive energy balance for the duration of the experiment (3.2 ± 0.66 Mcal/d of NE_L), limiting caloric intake in FR cows induced a NEB of -14.1 ± 0.62 Mcal/d of NE_L (Table 2). To compensate for reduced energy availability, cows in the FR group had a lipolytic response characterized by a progressive, marked increase in plasma NEFA concentrations over the course of the feed restriction period (Figure 1A). By d 4, FR increased NEFA concentrations to 0.65 mEq/L compared with 0.16 mEq/L

in AL group ($P < 0.01$). Lipolytic activity was demonstrated in OM and SC adipose depots by increased phosphorylation of HSL at Ser⁶⁶⁰ (Figure 1B). As expected, OM adipocytes were larger than SC before and after treatment (Figure 1C). Reflecting enhanced lipid mobilization, FR reduced adipocyte diameter in OM and SC adipose compared with AL (Figure 1C). The FR tended to have a lower concentration of plasma insulin by d 4 compared with AL ($P = 0.07$); however, circulating glucose concentrations remained unaltered (Figure 1D). In response to 4 d of caloric restriction, the expression of lipogenic genes *FASN*, *SCD1*, and *ELOVL6*, and the lipolytic gene *ABHD5* ($P < 0.05$) were reduced in OM and SC adipose. The transcription of the gene encoding ATGL (*PNPLA2*; $P = 0.08$) tended to be downregulated in both tissues from FR. Although FR did not alter the expression of fatty acid transporter *FABP4*, the treatment $\times$ site interaction for this gene was significant ($P = 0.04$). The FR had no effect on the transcription of *LIPE* that encodes HSL and the adipogenic regulator gene *PPARG* (Table 3).

NEB Reduces Milk Production and Alters Composition

As expected, milk yield, ECM, and FCM were reduced in the FR group after 4 d of limiting feed intake (Table 2). In contrast, FR increased fat yield ($P = 0.03$) and tended to elevate milk fat concentration ($P = 0.09$). Higher producing cows exhibited a stronger increase in fat yield and milk fat concentration ($P = 0.04, 0.06$), but a larger decrease in ECM and FCM in response to FR ($P = 0.07, 0.09$; Table 2). Reducing caloric intake negatively affected milk protein yield ($P < 0.01$). However, no differences were observed in milk protein concentration. Similarly, FR reduced lactose yield and concentration ($P < 0.01$ and $P = 0.02$, respectively). No differences were found in milk, fat, and lactose production or concentration responses to FR between blocks 1 and 2.

NEB Alters ATM Trafficking, But Does Not Affect Phenotype

The qPCR analysis of OM and SC adipose revealed that the expression of M1-related gene *TNFA* tended to be upregulated by FR ($P = 0.06$; Table 3). In contrast, expression of M1 related pro-inflammatory gene *CCL2* was not affected by caloric restriction. Though FR did not alter the expression of *ITGAX*, a gene encoding the M1 related surface protein CD11c, the treatment $\times$ site interaction for this gene was significant ($P = 0.04$). Transcriptions of M2-related anti-inflammatory genes *ARG1* and *IL10* were not affected by treatment

($P = 0.11$ and $P = 0.22$, respectively). No treatment or site effects were observed in the expression of the M2-related gene *CCL22*. The expression of *SIRPA*, a marker of mononuclear immune cells, was decreased by FR and its expression was lower in OM adipocytes of both AL and FR, compared with SC ($P = 0.03$). Based on flow cytometry analysis, FR increased the number of cells expressing the mononuclear cell marker CD172a (Table 4). The expression of CD14 and CD11c, both M1 markers, were unaltered. The FR did not have an effect on the number of CD11c⁺ cells in OM or SC; however, the treatment by site interaction for this marker was significant ($P = 0.05$; Table 4). More B cells were detected in OM fat than in SC fat; however, no effect of treatment was found (Table 4). Immunohistochemical analysis of OM and SC AT sections using anti-CD172a, a mononuclear immune cell specific marker, was used to determine ATM localization. The FR increased the number of ATM aggregates compared with AL ($P = 0.02$); however, the number of aggregates was not affected by site ($P = 0.07$; Figure 2). Notably, ATM aggregates did not contain lipid remnants or exhibit multinucleated cells.

ECMT Gene Expression Varies by AT Site But Is Not Affected by NEB

Expression of ECMT protein genes *COL1A1* and *COL6A2* was higher in SC than OM adipose ($P = 0.01$ and $P < 0.01$, respectively); however, they were not affected by treatment (Table 3). In contrast, the

expression of *THBS1* was similar in both sites and was not influenced by treatment. Finally, no effect of treatment was observed on the expression of cell cycle genes *CCNA2*, *CCNB2*, and *MKI67*, indicating no mitogenic effects. The expression of *SPP1* and *CD44*, the osteopontin and osteopontin receptor encoding genes, was not affected by treatment or site.

DISCUSSION

Although excessive lipolysis is a predisposing factor to disease and poor lactation performance in transition and early lactation cows, the number of confounding variables associated with parturition and the onset of lactation limit the interpretation of a single physiological process, such as lipolysis, on AT responses to NEB. Several research groups recognized this problem and designed experiments using mid- and late-lactation cows where early lactation NEB or lipolytic conditions are mimicked (Bjerre-Harpøth et al., 2012; Akbar et al., 2013; Pires et al., 2016). The main advantage of feed restriction in lipolysis models is that energy balance can be adjusted to mimic the transition period (Faylon et al., 2014). In our present study, we were able to emulate several metabolic features of transition dairy cows' metabolic function. First, plasma NEFA concentrations were increased in FR cows to simulate circulating NEFA concentrations observed in transition dairy cows. This lipolytic response was similar to that reported in other studies using 4-d feed restriction protocols (Faylon et al., 2014; Pires et al., 2016). Second, lipase activity at

Table 2. The effect of feed-restriction-induced negative energy balance and lipolysis on DMI, yield of milk and milk components, and energy balance of dairy cows¹

Variable	Treatment		P-value			
	Ad libitum	Restricted	Treatment	Block	pMilk ²	pMilk × treatment
DMI (kg/d)	25.7 ± 0.67	13.7 ± 1.42	0.01	NS ³	0.09	0.09
Yield (kg/d)						
Milk	35.7 ± 0.60	26.2 ± 0.56	<0.001	NS	NS	NS
ECM	36.6 ± 1.27	33.1 ± 2.71	0.04	NS	NS	0.09
Fat	1.3 ± 0.06	1.4 ± 0.13	0.03	NS	0.04	0.04
Protein	1.2 ± 0.04	0.8 ± 0.03	<0.001	NS	NS	NS
Lactose	1.7 ± 0.03	1.2 ± 0.03	<0.001	NS	NS	NS
Milk composition (%)						
Fat	3.8 ± 0.16	5.2 ± 0.34	0.10	NS	NS	0.06
Protein	3.3 ± 0.10	3.2 ± 0.09	0.27	NS	NS	NS
Lactose	4.7 ± 0.04	4.6 ± 0.04	0.02	NS	NS	0.03
Energy balance (Mcal/d of NE _L)	3.2 ± 0.66	-14.1 ± 0.62	<0.001	NS	0.03	NS

¹Late lactation dairy cows (n = 13) were randomly assigned to 1 of 2 feeding protocols: ad libitum (AL, n = 6) or feed-restricted (FR, n = 7). Caloric intake was reduced in FR cows for 4 d to achieve a targeted negative energy balance of -15 Mcal/d of NE_L with the quantity of TMR provided to each cow adjusted on d 3 of the feed restriction period to account for changes in milk production. Data are least squares means ± SEM.

²pMilk = preliminary milk yield.

³NS = $P > 0.10$.

Figure 1. Feed-restriction-induced negative energy balance increases lipolysis in lactating dairy cows. Lipolysis markers including plasma nonesterified fatty acids (NEFA; A), phosphorylation ratio (at Serine⁶⁶⁰) of hormone sensitive lipase (HSL) in omental (OM) and subcutaneous (SC) adipose depots (B), adipocyte diameters in OM and SC fat depots (C), and plasma insulin and glucose (D) were evaluated in late-lactation dairy cows. pHSL = rabbit anti-mouse phosphorylated HSL serine 660. Animals were randomly assigned to ad libitum (AL, $n = 6$) or feed-restricted (FR, $n = 7$) feeding protocols for 4 d to achieve a targeted negative energy balance of -15 Mcal/d of NE_L. Data are LSM $\pm$ SEM. Significant differences are indicated by **($P < 0.01$) and tendencies are indicated by *($0.05 < P < 0.10$).

the lipid droplet level increased in FR cows, reflected by enhanced HSL phosphorylation at Ser⁶⁶⁰ (Choi et al., 2010), and mimicked lipase activity during the first

2 wk after parturition (Koltes and Spurlock, 2011). Third, lipogenic gene networks were downregulated in FR cows, including fatty acid synthase (*FASN*) and

Table 3. The effect of feed-restriction-induced negative energy balance and lipolysis on the expression of genes related to lipid mobilization, extracellular matrix, and adipose tissue macrophage (ATM) phenotype and inflammation of dairy cows¹

Network	Gene	Fold change relative to ad libitum subcutaneous ^{2,3}				P-value		
		Ad libitum		Restricted		Treatment	Site	Treatment × site
		Subcutaneous	Omental	Subcutaneous	Omental			
Lipid mobilization	<i>ABHD5</i>	1 ^{ab}	1.44 ^a	0.82 ^{bc}	0.55 ^c	<0.01	NS ⁴	0.03
	<i>ELOVL6</i>	1	4.06	0.20	0.20	<0.01	NS	0.09
	<i>PNPLA2</i>	1	2.08	0.91	0.70	0.09	NS	NS
	<i>SCD1</i>	1	1.17	0.20	0.04	<0.01	NS	NS
	<i>FASN</i>	1	1.64	0.17	0.09	<0.01	NS	NS
	<i>FABP4</i>	1	1.78	2.07	1.05	NS	NS	0.04
	<i>LIPE</i>	1	2.02	2.06	1.13	NS	NS	NS
	<i>PPARG</i>	1	1.65	1.05	0.81	NS	NS	NS
Extracellular matrix	<i>COL1A1</i>	1	0.19	0.76	0.33	NS	<0.01	NS
	<i>COL6A2</i>	1	0.30	0.87	0.46	NS	<0.01	NS
	<i>THBS1</i>	1	0.44	0.78	1.02	NS	NS	NS
	<i>CCNA2</i>	1	1.15	0.90	1.44	NS	NS	NS
	<i>CCNB2</i>	1	1.23	0.83	1.14	NS	NS	NS
	<i>MKI67</i>	1	0.75	0.81	1.13	NS	NS	NS
	<i>SPP1</i>	1	0.72	0.57	0.62	NS	NS	NS
	<i>CD44</i>	1	0.47	0.65	0.58	NS	NS	NS
	<i>ARG1</i>	1	2.08	2.56	4.88	0.12	NS	NS
	<i>CCL2</i>	1	0.52	0.93	0.83	NS	NS	NS
ATM phenotype inflammation	<i>CCL22</i>	1	0.34	0.46	0.81	NS	NS	NS
	<i>IL6</i>	1	0.50	0.71	0.88	NS	NS	NS
	<i>IL10</i>	1	0.76	1.48	1.10	NS	NS	NS
	<i>ITGAX</i>	1 ^a	0.46 ^{ab}	0.23 ^b	0.70 ^{ab}	NS	NS	0.04
	<i>TNFA</i>	1	0.75	1.54	1.13	0.06	NS	NS
	<i>SIRPA</i>	1	0.58	0.57	0.41	0.03	0.03	NS

^{a-c}Fold changes without a common superscript within a row represent differences determined by *t*-tests of the ΔCt values.

¹Late-lactation dairy cows ($n = 13$) were randomly assigned to 1 of 2 feeding protocols: ad libitum (AL, $n = 6$) or feed-restricted (FR, $n = 7$). Caloric intake was reduced in FR cows for 4 d to achieve a targeted negative energy balance of -15 Mcal/d of NE_L with the quantity of TMR provided to each cow adjusted on d 3 of the feed restriction period to account for changes in milk production. Data are presented as fold changes relative to the ad libitum subcutaneous sample.

²Fold change = $2^{(-\Delta\Delta\text{Ct})}$; $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{calibrator sample}} - \Delta\text{Ct}_{\text{target sample}}$.

³Gene expression fold changes were calculated from LSM differences of the ΔCt values ($\Delta\Delta\text{Ct}$) normalized to the mean of *EIF3K*, *B2M*, and *RPS9* housekeeping genes. The ad libitum subcutaneous sample was the calibrator for the calculation of $\Delta\Delta\text{Ct}$. 95% confidence intervals of the $\Delta\Delta\text{Ct}$ values were transformed to fold changes and are presented in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2016-11552>).

⁴NS = $P > 0.10$.

Table 4. The effect of feed-restriction-induced negative energy balance and lipolysis on immune cell trafficking in omental and subcutaneous adipose tissues of dairy cows¹

Surface marker ²	Ad libitum		Restricted		P-value		
	Subcutaneous	Omental	Subcutaneous	Omental	Treatment	Site	Treatment × site
CD172a	12.31 ± 1.28	8.73 ± 1.28	13.83 ± 1.28	13.65 ± 1.28	0.02	NS ³	NS
CD14	4.08 ± 0.86	4.42 ± 0.86	4.82 ± 0.86	5.10 ± 0.86	NS	NS	NS
CD11b	4.37 ± 1.07 ^{ab}	3.45 ± 1.07 ^a	3.42 ± 1.07 ^a	6.93 ± 1.07 ^b	NS	NS	0.05
CD11c	1.92 ± 0.92	3.77 ± 0.73	4.17 ± 0.73	4.12 ± 0.73	NS	NS	NS
CD163	2.44 ± 0.46	2.75 ± 0.54	2.77 ± 0.46	2.79 ± 0.60	NS	NS	NS
CD3	3.83 ± 0.61	4.28 ± 0.61	3.88 ± 0.61	4.27 ± 0.61	NS	NS	NS
B cells	4.17 ± 1.39	7.98 ± 1.07	5.48 ± 1.07	7.52 ± 1.07	NS	0.02	NS

^{a,b}Least squares means without a common superscript within a row represent differences determined by *t*-tests.

¹Late-lactation dairy cows ($n = 13$) were randomly assigned to 1 of 2 feeding protocols: ad libitum ($n = 6$) or feed-restricted ($n = 7$). Caloric intake was reduced in feed-restricted cows for 4 d to achieve a targeted negative energy balance of -15 Mcal/d of NE_L with the quantity of TMR provided to each cow adjusted on d 3 of the feed restriction period to account for changes in milk production.

²Values are least squares means of percentages of stromal vascular fraction cells expressing phenotype surface markers ± SEM as measured by flow cytometry using specific bovine monoclonal antibodies.

³NS = $P > 0.10$.

Figure 2. Adipose tissue macrophage (ATM) aggregation is increased in cows during feed-restriction-induced negative energy balance. The ATM localization was evaluated by immunostaining of omental (OM) and subcutaneous (SC) adipose tissue sections from late-lactation dairy cows after 4 d of an ad libitum (AL, $n = 6$) or feed-restricted (FR, $n = 7$) feeding protocol. (A) High and low magnification images of SC and OM adipose tissues and representative images of spleen, representing a positive tissue control, from a FR cow. Black arrows indicate immunoreactive CD172a macrophages. Scale bars = 50 μm . (B) Number of ATM aggregates per 100 adipocytes in OM and SC adipose tissues of AL and FR cows. Data are LSM $\pm$ SEM. Color version available online.

elongase (*ELOVL6*). This occurs in transition dairy cows in response to hypoinsulinemia and lipolysis and is exacerbated in cows of high genetic merit (Khan et al., 2013). Finally, our results also emulate periparturient metabolic status because of hypoinsulinemia (De Koster and Opsomer, 2013). Interestingly, our feed restriction model did not induce hypoglycemia, a common observation in transition dairy cows. Previous feed restriction studies have reported similar results (Velez and Donkin, 2005; Oikawa and Oetzel, 2006), whereas others indicated a reduction in plasma glucose (Gross et al., 2011b; Ferraretto et al., 2014). Divergent results may be related to the DIM at the time of feed restriction because studies with cows around 100 DIM had lower circulating glucose compared with cows >150 DIM (Gross et al., 2011b). Additionally, a reduction in glycemia may be mitigated by gluconeogenesis from propionate, lactate, or glycerol. The latter is an important lipolysis product in animals during NEB (Herdt, 2000; Reynolds et al., 2003).

Previous studies found that NEB and lipolysis alter milk composition and production (Palmquist et al., 1993). Higher availability of circulating NEFA is rapidly reflected in milk fat as the mammary gland actively utilizes plasma NEFA for milk fat synthesis (Pullen et al., 1989). Higher milk fat is characteristic during the first week after calving and is greatly affected by the intensity and duration of lipolysis as a NEB response (van Knegsel et al., 2007; Mann et al., 2015). Similar to other feed restriction studies in early (Nielsen et al., 2003) and mid lactation (Gross et al., 2011a) cows, NEB-induced lipolysis in FR cows increased milk fat yield and concentration in our study, reflecting higher availability of circulating fatty acids to the mammary gland. Interestingly, the increase in fat concentration and yield due to FR was positively associated with milk production before feed restriction. Although plasma NEFA concentrations during FR were not affected by milk production before caloric restriction, it is unclear if lipolytic responses to NEB in late lactation are affected by previous levels of milk production, as has been reported in early lactation cows (Khan et al., 2013). Although conjecture, a higher lipolytic response to NEB in high producing cows in late lactation may be reflected in higher milk fat yield and concentration, but similar plasma NEFA compared with lower producing cows.

Transition cow AT inflammation is characterized by higher gene expression of pro-inflammatory cytokines including TNF α and IL-6 (Sadri et al., 2010; Schmitt et al., 2011). In our group of late-lactation cows, only *TNFA* gene expression was upregulated in response to lipolysis and no changes were observed in the transcript of additional cytokines considered early markers of in-

flammation in dairy cattle such as IL-6 and macrophage chemoattractant protein 1 (encoded by *CCL2*; Rainard et al., 2013). To our knowledge, only one previous study analyzed inflammatory responses during caloric restriction (Perkins et al., 2002). In that study circulating and mammary inflammatory markers were evaluated in mid-lactation cows after an intramammary LPS challenge. No differences were found in the inflammatory response between FR (80% of energy requirements) and AL-fed cows. In that published study and our current study, NEFA concentrations and NEB values were similar to those observed around parturition; however, the duration of NEB was shorter than what is observed in transition cows. Thus, to adequately evaluate the effect of lipolysis on inflammatory responses in AT or other organs using late lactation models of transition cow NEB, it may be necessary to extend caloric restriction interventions to several weeks in duration.

Lipolysis partly induces ATM infiltration by excessive release of fatty acids from adipocytes (Kosteli et al., 2010). Following migration into adipose, ATM uptake lipid as an adaptive response to excessive adipocyte lipolysis that prevents lipotoxicity at the adipose and systemic level (Prieur et al., 2011). In transition dairy cows, Akter et al. (2012) reported some ATM infiltration in OM and mesenteric AT of early lactation heifers, but not in SC AT. In that study, plasma NEFA and NEB were similar to those observed in our FR cows. In line with their findings, our results indicate ATM infiltration in OM adipose. Thus, in transition cows and during intense short lipolytic events, such as our caloric restriction model, lipolysis induces a mild ATM infiltration into adipose depots. Although a higher number of infiltrating ATM localized in aggregates were identified in FR cows, these cell conglomerates did not exhibit multinucleated cells or lipid remnants as was observed previously in transition cows with DA (Contreras et al., 2015). Furthermore, the number of ATM aggregates/100 adipocytes was below 1 compared with over 2 ATM aggregates/100 adipocytes in cows with DA (Contreras et al., 2015). These differences in the degree of ATM infiltration and aggregation may be related in part to the limited adipocyte turnover (i.e., apoptosis and necrosis) in FR cows that was reflected in the low expression of mitogenic genes. In monogastric species, adipocyte apoptosis and necrosis are important triggers of ATM aggregation and M1 polarization (Martinez-Santibañez and Nien-Kai Lumeng, 2014). The lower number of ATM aggregates in FR cows may also be linked to the relatively short duration of induced NEB (4 d). It is probable that longer periods of NEB increase the accumulation of lipolysis products in AT and further increase ATM aggregation over levels seen in this study. Identifying biomarkers of ATM ag-

gregation in AT may provide a better assessment of AT metabolic function in transition dairy cows.

Lipolysis is also recognized as a modulator of ATM inflammatory phenotype. In rodents, extended periods of lipolysis polarize ATM toward a M1 (pro-inflammatory) phenotype (Kosteli et al., 2010). In transition dairy cows, we previously demonstrated that extended periods of NEB with high lipolytic rates associated with DA increase ATM populations and induce M1 phenotype polarization (Contreras et al., 2015). In our present study, however, cows in NEB did not exhibit increased expression of M1-related genes or cell surface proteins indicating changes in their inflammatory phenotype. Future studies should assess the effect of extended periods of lipolysis (several weeks) and also parturition-related physiological changes that are independent of lipolysis that may affect ATM infiltration and phenotype.

To our knowledge, this is the first study that evaluated gene expression of ECMT collagens (I and VI) and thrombospondin in AT from dairy cows during NEB. In humans and rodent models of insulin resistance, excessive deposition of these collagens in AT enhances macrophage infiltration (Khan et al., 2009; Divoux et al., 2010; Guglielmi et al., 2015). Also, in diabetic humans, collagen VI limits adipocyte expansion and therefore fatty acid uptake (Divoux et al., 2010). Our results indicate higher expression of both collagens in SC fat compared with OM fat during NEB. This finding highlights the differences in ECMT composition depending on the specific anatomical site of AT depots. It is possible that intense lipolysis for several weeks in the transition period may induce changes in the composition of ECMT that could impair AT function. Thus, determining the patterns of adipose ECMT change during the transition period may identify novel markers of AT function.

Our feed restriction protocol and interpretation of results have limitations. First, although we were able to mimic the lipolysis intensity observed around parturition, aspects of metabolic regulation critical to AT function are unique to the transition cow and cannot be mimicked by a restricted feeding model. For example, our feed restriction scheme did not emulate the duration of the lipolytic stimuli of transition dairy cows that may last up to 6 to 8 wk. It is possible that longer periods of intense lipolysis may have a more profound effect on AT remodeling features such as ATM infiltration and local inflammatory responses. A second limitation is that mid- and late-lactation cows adapt to NEB, not only by mobilizing lipid reserves from adipose, but also by reducing milk production. Although this mechanism reflects the differences in physiological profiles of transition and late-lactation cows, it provides a reductionist

model where NEB-induced lipolysis is evaluated as a single factor in the modulation of AT remodeling.

CONCLUSIONS

Elevated rates of lipolysis during the transition period may lead to an acute AT remodeling process that could impair AT capacity for buffering excessive concentrations of circulating NEFA and therefore increase the risk for metabolic and inflammatory diseases. During severe NEB in transition cows with DA, AT remodeling is characterized by ATM infiltration and M1 polarization; however, the direct effect of lipolysis in NEB as a single physiological factor was unknown. Using a caloric restriction approach in late-lactation dairy cows that induced a NEB similar to that observed in transition dairy cows, we demonstrate for the first time that a 4-d intense lipolytic stimulus led to ATM infiltration in AT but with only minimal changes in inflammatory phenotype. These results underscore that physiological changes related to parturition and the onset of lactation or extended periods of lipolysis (or both) may induce intense AT remodeling with enhanced ATM inflammatory phenotype expression that may impair the metabolic function of AT in transition dairy cattle. Further studies are required to elucidate the dynamics of AT cellular and ECMT responses during AT remodeling in healthy transition dairy cows and the period of NEB required to impair the metabolic function of AT in dairy cattle.

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