Adipose tissue lipogenic gene networks due to lipid feeding and milk fat depression in lactating cows

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ABSTRACT

Dietary lipid supplements have been extensively evaluated for their effects on mammary tissue mRNA abundance, including the classical lipogenic genes *ACACA*, *SCD*, *FASN*, and the transcription regulators *SREBF1*, *THRSP*, and *PPARG*. Novel gene isoforms with key regulatory roles in triacylglycerol synthesis have been recently identified including *LPIN1* and *AGPAT6*. Transcriptional networks (i.e., genes whose mRNA expression is regulated by a transcription factor or nuclear receptor) coordinate adipogenesis and lipid filling in nonruminant adipose tissue. To investigate whether long-term milk fat depression affects adipogenic networks in subcutaneous adipose tissue, we characterized mRNA expression via quantitative PCR of 20 genes in cows fed saturated and polyunsaturated lipid for 3 wk. Adipose tissue from cows fed a control diet, control with fish (10 g/kg of dry matter) and soybean oil (25 g/kg of dry matter) (FSO), or control with saturated lipid (35 g/kg, EB100; Energy Booster 100, Milk Specialties, Dundee, IL) was biopsied after 21 d of feeding. Milk production did not differ across treatments (averaged 32 kg ± 2.8 kg/d during the 21 d) but dry matter intake (DMI) decreased in cows fed FSO versus controls (averaged 18 vs. 22 kg/d during the 21 d). Despite the decrease in DMI, FSO resulted in similar energy intake as EB100 during the last 2 wk of the study. Cows fed FSO had a gradual decline in milk fat and energy yield leading to an overall 25% decrease in milk fat yield during the study (averaged 0.90 vs. 1.2 kg/d) compared with control or EB100. Thus, during the 21-d study, FSO led to a gradual increase in intake energy available for adipose tissue deposition. Relative mRNA expression of *LPL* and *SCD* as well as *ADFP* (coding for a protein involved in lipid droplet formation) and *LPIN1* (coding for a protein involved in diacylglycerol synthesis/transcriptional regulation) was upregulated with FSO relative to other diets. Expression of the transcription regulator *THRSP* tended to be

greater in cows fed FSO. Overall, results suggest that long-term milk fat depression caused by feeding FSO provided additional energy as well as long-chain fatty acids that, coupled with upregulation of a subset of adipogenic genes in subcutaneous adipose tissue, might have resulted in greater tissue lipid deposition.

Key words: transcriptomics, nuclear receptor, transcription regulator

INTRODUCTION

Well-defined effects of diet-induced milk fat depression include lower mammary mRNA abundance of the classical lipogenic genes acetyl-CoA carboxylase (*ACACA*), fatty acid synthase (*FASN*), and stearoyl-CoA desaturase (*SCD*; Peterson et al., 2004), as well as the transcription factor sterol regulatory element binding factor 1 (*SREBF1*; Harvatine and Bauman, 2006). In recent years, a myriad of genes with apparently key regulatory roles in the synthesis of triacylglycerol (**TAG**) have been identified. Lipin 1 (phosphatidic acid phosphatase, *LPIN1*) and 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*) are 2 examples of genes coding for enzymes of the TAG pathway, which are essential in adipose (Reue and Zhang, 2008) and mammary gland of rodents (Beigneux et al., 2006). In addition, *LPIN1* acts as a co-activator of peroxisome proliferator-activated receptor (**PPAR**) in liver and potentially in adipose tissue of rodents (Reue and Zhang, 2008).

Studies in nonlactating sheep and lactating cows showed that short-term (hours to a few days) manipulation of nutrients [e.g., propionate or conjugated linoleic acid (**CLA**)] available to the animal can have substantial effects on subcutaneous adipose tissue (**SUBQ**) gene expression (Lee and Hossner, 2002; Harvatine et al., 2009). Based on those data, it is likely that long-term nutritional regulation of milk fat synthesis during lactation involves not only transcriptomic adaptations in mammary tissue but also in adipose tissue. Milk production, fat composition and yield, and **SUBQ** rates of lipogenesis and esterification are affected by stage of lactation and supplemental lipid (McNamara et al., 1995).

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Metabolic regulation in complex organisms relies partly on transcriptional control as a long-term mechanism affecting the level of expression of several key enzymes (Desvergne et al., 2006). A cellular gene network can be defined as a collection of DNA segments that interact with a regulator such as a transcription factor or nuclear receptor, but also with each other through their RNA and protein products and with other molecules in the cell (Wittkopp, 2007). These “global” interactions, thus, can govern the rates at which genes in the network are transcribed into mRNA. In rodents, there is high correlation between mRNA expression of target genes and recruitment of lipogenic transcription factors or nuclear receptors and their co-regulatory proteins to promoter regions, suggesting that gene expression analysis is useful for inferring transcriptional activity (Bennett et al., 2008). Transcriptional regulation of gene networks involved in hepatic lipogenesis and adipogenesis in adipose tissue of rodents is under control of sterol regulatory element binding factor 1 (*SREBF1*) (Horton et al., 2002) and the ligand-activated nuclear receptor PPAR γ (*PPARG*), respectively (Fernyhough et al., 2007).

As a means to evaluate long-term metabolic regulation, our group has recently studied transcriptional networks involved in mammary (Bionaz and Loor, 2008a,b) or skeletal muscle tissue (Graugnard et al., 2009) during lactation or rapid growth. In the course of similar studies of long-term nutritional regulation of milk fat synthesis (Thering, 2008), we sought to examine transcriptional changes of adipogenic and lipogenic networks in SUBQ. Our hypothesis was that long-term diet-induced milk fat depression in response to a blend of fish and soybean oil would be associated with greater mRNA abundance of tail-head SUBQ lipogenic or adipogenic gene networks (Figure 1). Specific objectives were to measure mRNA abundance of 20 genes encoding proteins required for fatty acid uptake and activation, intracellular fatty acid transport, de novo fatty acid synthesis, esterification, desaturation, transcriptional regulation of adipogenesis and differentiation, and lipid droplet formation.

MATERIALS AND METHODS

Animals, Management, Diets, and Sampling

Sixteen Holstein cows from the University of Illinois dairy herd were used during a 4-wk study. Cows averaged 100 ± 13 DIM, 41.7 ± 6.49 kg of milk/d, $3.99 \pm 0.66\%$ fat, $2.90 \pm 0.27\%$ protein, and 2.70 ± 0.31 BCS before the beginning of the study. Before assignment to diets, cows were first blocked based on previous-week average milk yield, milk fat percentage, and BCS. Cows

were then assigned to treatment groups based on milk production level (first criterion), milk fat percentage (second criterion), and BCS (third criterion) in an attempt to achieve homogeneous groups. Before commencing the study, average milk production for the 3 groups was 41.3, 42.6, and 41.3 kg/d; average milk fat percentage for the 3 groups was 3.83, 3.95, and 4.03; and average BCS for the 3 groups was 2.6, 2.7, and 2.7. Cows were housed in individual tie stalls bedded with sawdust and were offered a total mixed diet once daily (1100 h). Cows had continuous access to water and were milked at 0500 and 1600 h throughout the study.

Diets were based on corn silage and alfalfa silage (Thering, 2008). Treatments included a control diet with no added lipid ($n = 5$ cows), a milk fat-depressing diet containing a blend of fish oil (10 g/kg; Omega Protein Inc., Houston, TX) and soybean oil (25 g/kg; Archer Daniels Midland, Decatur, IL; $n = 5$ cows, **FSO**), and a diet containing a saturated lipid supplement (Energy Booster 100, Milk Specialties, Dundee, IL; 35 g/kg of DM; $n = 6$ cows, **EB100**). Diets had a forage:concentrate ratio of 70:30 (DM basis), with lipid supplements in FSO and EB100 replacing soybean hulls. Ingredient and chemical composition of experimental diets were reported previously (Thering, 2008). Intake of NE_L was calculated as $DMI \text{ (kg/d)} \times NE_L \text{ (Mcal/kg of DM)}$ content of each diet (1.55, 1.66, and 1.67, respectively, for control, FSO, and EB100). Milk NE_L output (Mcal/d) was calculated as $milk \text{ yield (kg)} \times [0.0929 \times (\text{fat } \%) + 0.0563 \times (\text{true protein } \%) + 0.0395 \times (\text{lactose } \%)]$ (NRC, 2001). Diets were fed ad libitum as a TMR.

Individual BW were recorded weekly; individual feed intake was recorded daily. Samples of fresh TMR, feed ingredients, and feed refusals were collected weekly and stored at -20°C . At the end of the experiment, feed samples were oven-dried (110°C) for 24 h and DM percentage determined.

Milk yields were electronically recorded at each milking. Samples of milk for the determination of fat, protein, and lactose were collected from each cow at 0500 and 1600 h each day (total of 56 individual samples). Composite samples of milk (28 total) from a.m. and p.m. milkings were obtained by combining a volume of a.m. or p.m. sample based on the proportion of milk produced at each milking. Samples were stored with Bronopol preservative (D&F Control Systems Inc., Dublin, CA) at 4°C before milk composition analysis by mid-infrared methods (AOAC, 1995) at a DHIA laboratory (Dairy One, Ithaca, NY).

Blood samples from the coccygeal artery/vein were collected before the morning feeding on d 21 of feeding the diets. Samples were analyzed to determine concentrations of NEFA, BHBA, TAG, and glucose using

Figure 1. Currently known relationships among genes analyzed based on manually curated examination of the published literature within the Ingenuity Pathway Analysis (www.ingenuity.com) knowledge base. Genes are grouped by the predominant process they play in lipid metabolism. Different shapes denote the type of protein encoded by the specific genes, including enzymes, ligand-dependent nuclear receptors, transcription regulators, and transporters. The type of relationships connecting genes are indicated by a capital letter according to the legend in the figure. Gene names are as in Table 1.

commercial kits in an autoanalyzer at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL).

Biopsies

On d 21 relative to start of treatments, and following an approved Institutional Animal Care and Use Committee protocol, the tail-head area of all cows was evaluated visually and by palpation to determine if sufficient amounts of SUBQ could be harvested without the need for extensive dissection around the incision, which may have resulted in excessive trauma to the chosen area or excessive pain/distress to the animal. An additional concern was the fact that, in our experience, the yield of total RNA per gram of SUBQ is markedly lower compared with that from tissues such as liver and mammary. It was not expected that the cow selection approach would confound our analysis because the chosen cows responded to dietary treatments in the same fashion (Table 1) as the entire group (Thering, 2008). Based on the above criteria, it was deemed that only 3 cows per treatment were adequate for biopsies. Before biopsies, the hair on the tail-head and to one side of the

tail-head was clipped closely and thoroughly scrubbed with surgical soap. Local anesthesia was applied over the area between the point of the ischium and coccygeal vertebra. A 6- to 8-cm incision was made and the skin pulled back using sterile forceps, exposing the SUBQ. Samples were taken using a sterile scalpel blade and forceps. After the sample was taken, pressure was applied with sterile gauze to stop any external bleeding. The incision was closed with 8 to 12 surgical staples (#89063337, Appose ULC Skin Stapler, 35 wide, Henry Schein Inc., Melville, NY) and iodine ointment was applied. Biopsied tissue (3–5 g) was weighed and stored in liquid N₂ before RNA extraction.

RNA Extraction, Primer Design and Evaluation, and Quantitative PCR

The RNA was extracted using QIAzol Lysis Reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols. Genomic DNA was removed from RNA during the last step of extraction with DNase (Qiagen, Hilden, Germany). Then, RNA was resuspended in RNase-free water and stored at -80°C until used. The concentration of RNA was measured using a NanoDrop

Table 1. Gene symbol, cellular location, and main biological process¹ of the 20 genes measured in subcutaneous adipose tissue

Gene symbol	Description	Cellular localization	Biological process
<i>ACACA</i>	Acetyl-coenzyme A carboxylase α	Cytoplasm	Fatty acid biosynthesis
<i>ACSL1</i>	Acyl-CoA synthetase long-chain family member 1	Plasma and endoplasmic reticulum (ER) membranes	Fatty acid metabolism
<i>ACSS2</i>	Acyl-CoA synthetase short-chain family member 2	Cytosol, cytoplasm	Lipid biosynthesis
<i>ADFP</i>	Adipose differentiation-related protein	Cytoplasm, endoplasmic reticulum	Long-chain fatty acid transport, sequestration of lipid
<i>AGPAT6</i>	1-Acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransferase, zeta)	ER membrane	Phosphatidic acid biosynthesis
<i>CD36</i>	CD36 molecule (thrombospondin receptor)	Plasma membrane	Fatty acid metabolism
<i>DGAT1</i>	Diacylglycerol O-acyltransferase homolog 1	ER membrane	Triacylglycerol metabolism
<i>FABP3</i>	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	Cytosol	Fatty acid binding, transport
<i>FABP4</i>	Fatty acid binding protein 4, adipocyte	Cytosol	Fatty acid binding, transport
<i>FADS1</i>	Fatty acid desaturase 1	ER membrane	Unsaturated fatty acid synthesis
<i>FASN</i>	Fatty acid synthase	Cytosol	Fatty acid biosynthesis
<i>GPAM</i>	Glycerol-3-phosphate acyltransferase, mitochondrial	Cytosol, mitochondria	Triacylglycerol biosynthesis
<i>INSIG1</i>	Insulin induced gene 1	ER membrane	Lipid metabolism, cell proliferation
<i>LPIN1</i>	Lipin 1	Nucleus, ER, cytosol	Phosphatidic acid hydrolysis, transcription
<i>LPL</i>	Lipoprotein lipase	Plasma membrane, extracellular region	Triacylglycerol metabolism
<i>PPARG</i>	Peroxisome proliferator activated receptor gamma	Nucleus	Induction of adipocyte differentiation, transcription
<i>SCAP</i>	SREBF chaperone	ER membrane, Golgi	SREBF target gene transcription activation
<i>SCD</i>	Stearoyl-CoA desaturase (Δ^9 -desaturase)	ER membrane	Fatty acid biosynthesis
<i>SREBF1</i>	Sterol regulatory element binding transcription factor (SREBF) 1	Golgi and ER membranes, nucleus	Transcription regulation
<i>THRSP</i>	Thyroid hormone responsive (SPOT14 homolog, rat)	Nucleus	Transcription regulation

¹Entrez Gene, National Center for Biotechnology Information (NCBI).

ND-1000 spectrophotometer (www.nanodrop.com); the purity of RNA (A_{260}/A_{280}) for all samples was approximately 2.0. A portion of the RNA was diluted to 100 ng/ μ L using DNase/RNase-free water before reverse transcription. Samples of RNA were stored at -80°C until use.

Complementary DNA was synthesized using 100 ng of RNA, 1 μ g of dT18 (Operon Biotechnologies, Huntsville, AL), 1 μ L of 10 mmol/L dNTP mix (Invitrogen Corp., Carlsbad, CA), 1 μ L of random primers (Invitrogen Corp.), and 10 μ L of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 6 μ L of master mix composed of 4.5 μ L of 5 $\times$ First-Strand Buffer, 1 μ L of 0.1 M dithiothreitol, 0.25 μ L (50 U) of SuperScript III RT (Invitrogen Corp.) and 0.25 μ L of RNase Inhibitor (10 U, Promega, Madison, WI) was added. The reaction was performed in an Eppendorf Mastercycler Gradient using the following temperature program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. Sample cDNA was then diluted 1:4 with DNase/RNase-free water, and cDNA for the standard curve was diluted 1:3 to ensure that it encompassed all levels of expression.

Quantitative PCR was performed using 4 μ L of diluted cDNA combined with 6 μ L of a mixture composed of 5 μ L of 1 $\times$ SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 μ L each of 10 μ M forward and reverse primers, and 0.2 μ L of DNase/RNase-free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). Quantitative PCR performance for all genes including internal controls is shown in Supplemental Table 1 (<http://jds.fass.org/content/vol92/issue9/>). Each sample was run in triplicate and a 5-point relative standard curve plus the nontemplate control were used (User Bulletin #2, Applied Biosystems). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 2 min at 50°C , 10 min at 95°C , 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were filtered to remove unreliable data and subsequently were normalized (5 to 9 replicates per diet) using the geometric mean

of ubiquitously expressed transcript (*UXT*), eukaryotic translation initiation factor 3, subunit K (*EIF3K*), and mitochondrial ribosomal protein L39 (*MRPL39*), which were identified as suitable internal controls among several tested (Kadegowda et al., 2009a).

Details on primer design, testing, sequencing, and actual primer features were reported previously (Bionaz and Loor, 2008a,b; Graugnard et al., 2009). Briefly, primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible amplicons of 100 to 150 bp were chosen) and limited 3' G+C (Applied Biosystems). When possible, primer sets were designed to fall across exon-exon junctions. Primers were aligned against publicly available databases using BLASTN at National Center of Biotechnology Information (National Center for Biotechnology Information, 2008) and UCSC's Cow (*Bos taurus*) Genome Browser Gateway (Genome Browser Gateway, 2008).

Gene Network Analysis

Summary networks among the chosen genes (Figure 1) were developed using the web-based software package Ingenuity Pathway Analysis (www.ingenuity.com; Redwood City, CA). The networks were generated using the respective gene identifiers and not the actual fold-changes in expression. Connections among genes were based on known relationships available in the Ingenuity Pathway Analysis knowledge base. This is a proprietary, manually curated database based on the published literature primarily in rodents and humans.

Statistical Analysis

All data were analyzed using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC). Normalized mRNA abundance data were log-transformed before statistical analysis. The model to examine treatment differences on mRNA abundance and blood serum metabolites consisted of treatment as fixed effect and cow within treatment as random effect. A similar model including treatment, time, and treatment $\times$ time as fixed effects and cow within treatment as random effect was used to determine differences in daily DMI, NE_L intake, milk NE_L yield, surplus NE_L intake, weekly BW, milk production, milk composition, and milk component yield. Least squares means (**LSM**) were separated using the PDIF statement in SAS.

RESULTS AND DISCUSSION

Responses in DMI, milk yield, milk fat yield (Figure 2), and milk fat percentage (Supplemental Figure 1; <http://jds.fass.org/content/vol92/issue9/>) due to FSO

compared with control or EB100-fed cows were similar to those reported previously for cows supplemented with a blend of fish (1.5% of DM) and sunflower oil (3.0% of DM) (Harvatine and Bauman, 2006; Shingfield et al., 2006). Weekly body weights, milk component concentrations, and yields of lactose and protein are reported in Supplemental Figures 1 and 2 (<http://jds.fass.org/content/vol92/issue9/>). A recent study found evidence that during short-term milk fat depression due to abomasal CLA infusion the energy spared from reduced milk fat synthesis was partitioned toward SUBQ and explained greater lipogenic mRNA abundance in this tissue relative to control animals (Harvatine et al., 2009). We also observed marked reductions in milk energy yield over time, which were accompanied by greater availability of intake energy potentially for adipose tissue deposition (Table 2, Figures 2 and 3). As suggested previously (Harvatine et al., 2009), responses in our study would likely reflect long-term adaptations by the animal to maintain body stores at a desired set point.

Few studies (Sumner and McNamara, 2007; Graugnard et al., 2009; Harvatine et al., 2009) have evaluated effects of nutrition on SUBQ or intramuscular adipose tissue gene expression in ruminants. After a 2-h propionate infusion into the jugular vein of nonlactating sheep, there was greater mRNA expression of *LPL*, *ACACA*, *FASN*, and *PPARG* (Lee and Hossner, 2002). We observed similar responses for *LPL* (a classical *PPARG* target gene; Rosen and MacDougald, 2006) and numerically greater *FASN* (an *SREBF1* target gene; Horton et al., 2002) mRNA when cows were fed FSO (Figure 4). Effects of propionate in sheep were probably indirect and mediated via greater glucose production in liver as well as the ensuing increase in blood insulin (Ferryhough et al., 2007). Similarly, reduced milk fat and energy yield coupled with greater lipogenic gene mRNA expression due to short-term CLA infusion would have allowed greater use of acetate, glucose, and long-chain fatty acids for lipogenesis and esterification in SUBQ (Harvatine et al., 2009).

In nonruminant liver and adipose tissue, glucose or high-carbohydrate diets (independently of insulin) can, in the short term, induce upregulation of *FASN*, pyruvate kinase (*PKLR*), and *ACACA* via the transcription factor *MLXIPL* (commonly referred to as carbohydrate-responsive element-binding protein, ChREBP); activation of *SREBF1* via insulin is also associated with the short-term upregulation of lipogenic gene transcription (Postic et al., 2007). Thus, in the fed state, lipogenesis and esterification in nonruminant liver and adipose tissue occur by the concerted action of glucose and insulin on the genes targeted by *SREBF1* and *MLXIPL*. In ruminants, greater *SREBF1* in SUBQ adipose could be

Figure 2. Daily DMI (A), milk production (B), milk fat yield (C), NE_L intake (D), milk NE_L yield (E), and surplus NE_L intake (F) due to feeding cows ($n = 3/\text{diet}$) a control diet, a milk fat-depressing diet containing fish oil (10 g/kg) and soybean oil (25 g/kg) (FSO), or a diet containing a saturated lipid supplement (EB100, 35 g/kg of DM; Energy Booster, Milk Specialties, Dundee, IL). Overall diet and treatment effects are shown in Table 2. Symbols denote interactions ($P < 0.05$) at the specified times: *control > FSO; #EB100 > FSO; \$control and EB100 > FSO; @control and EB100 > FSO; +EB100 > FSO; &control versus FSO or EB100 versus FSO.

associated with upregulation of lipogenic genes. However, it remains to be determined if *MLXIPL* plays any role in SUBQ as we have not detected expression of this gene in mammary tissue or mammary epithelial cells (Kadegowda et al., 2009a). Effects on SUBQ induced by milk fat depression due to dietary lipid supplementation are likely to be mediated both by the energy spared from reduced milk fat synthesis as well as direct effects of long-chain fatty acids on gene transcription via their binding to nuclear receptors such as PPAR γ .

Dietary fish oil increases the total fatty acid concentration in TAG of very low density lipoproteins (VLDL; Offer et al., 2001). However, because the saturated lipid supplement (EB100) in our study did not affect milk fat percentage or yield from controls (Table 2) it is likely

that greater adipose *LPL* mRNA with FSO was driven by reduced use of circulating TAG for milk fat production (approximately 26% lower fat yield vs. control or EB100; Table 2, Figure 2); that is, more VLDL was available for use by SUBQ. Under that scenario, there is no reason to believe that the change in *LPL* mRNA was not gradual and opposite to the pattern of milk fat and energy yield (Table 2, Figure 2); that is, over the 21-d period there was more TAG available for SUBQ utilization that might, in and of itself, have served as feed-forward mechanism for upregulation of *LPL*. Lipoprotein lipase activity and volume of adipocytes are substantially lower in SUBQ from lactating compared with dry/fattening cows (Chilliard and Robelin, 1985). The volume of subcutaneous adipocytes was not af-

Table 2. Body weight, DMI, milk yield and milk composition, estimated NE_L intake and output in milk, and arterial blood metabolites due to feeding (n = 3/diet) a control diet, a milk fat-depressing diet containing fish oil (10 g/kg) and soybean oil (25 g/kg) (FSO), or a diet containing a saturated lipid supplement (EB100, 35 g/kg of DM; Energy Booster, Milk Specialties, Dundee, IL)

Item	Treatment				P-value		
	Control	EB100	FSO	SEM	Diet	Time	Diet × time
BW (kg)	742	709	701	29	0.59	0.40	0.59
DMI (kg/d)	22.2 ^a	19.6 ^{ab}	17.7 ^b	0.7	0.01	0.01	0.01
NE _L intake (Mcal/d)	35.7 ^a	34.5 ^{ab}	31.7 ^b	0.9	0.04	0.01	0.01
Yield (kg/d)							
Milk	30.0	35.6	31.1	2.5	0.31	0.01	0.08
NE _L (Mcal/d)	21.2	23.7	19.2	1.6	0.22	0.01	0.01
Fat	1.15 ^{ab}	1.29 ^a	0.93 ^b	0.09	0.07	0.01	0.01
Protein	0.88	0.95	0.83	0.06	0.45	0.01	0.13
Lactose	1.41	1.60	1.48	0.12	0.54	0.01	0.14
Surplus NE _L intake ¹ (Mcal/d)	14.4	10.9	12.5	1.7	0.38	0.01	0.01
Percentage							
Fat	3.86 ^a	3.68 ^a	2.93 ^b	0.20	0.04	0.01	0.01
Protein	2.95	2.69	2.67	0.12	0.27	0.01	0.46
Lactose	4.72	4.51	4.72	0.14	0.52	0.01	0.18
Arterial blood serum							
Glucose (mg/dL)	67.3	68.7	66.3	2.7	0.84	—	—
BHBA (mg/dL)	0.57	0.47	0.57	0.09	0.66	—	—
Triacylglycerol (mg/dL)	14.3	17.0	12.3	0.45	0.45	—	—
NEFA (μEq/L)	0.29	0.33	0.25	0.11	0.85	—	—

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹NE_L intake (Mcal/d) – milk NE_L yield (Mcal/d).

affected greatly by dietary fat after peak lactation but it increased over the course of lactation (McNamara et al., 1995). Further, the capacity of SUBQ for lipogenesis from acetate and esterification of long-chain fatty acids also should be greater past peak lactation (McNamara et al., 1995). Thus, it is intuitive that a reduction in milk fat production that leads to a surplus of dietary energy past peak lactation would divert use of substrates for lipogenesis (acetate, BHBA) or direct esterification (VLDL-derived long-chain fatty acids) to adipose tissues.

Despite often reducing DMI, FSO increased ruminal organic matter and fiber digestibility (Wonsil et al., 1994; Doreau and Chilliard, 1997) as well as ruminal propionate concentration (Doreau and Chilliard, 1997). In addition, reduced milk fat synthesis with FSO previously led to lower mammary uptake of BHBA (approximately 60% from controls) without affecting its arterial plasma concentration (Lor et al., 2005), which also implies that this lipogenic precursor would be available to SUBQ for lipogenesis. In the present study, we did not observe changes in arterial concentrations of BHBA, NEFA, glucose, or TAG (Table 2). Although not statistically significant, the numerically greater *ACSS2* and *FASN* (Figure 4) as well as the greater capacity of SUBQ for lipogenesis at >120 DIM (McNamara et al., 1995) suggests that de novo synthesis might have been increased in cows fed FSO. The *ACSS2* gene encodes a cytosolic protein with high affinity for acetate and it

channels acetate toward fatty acid synthesis in nonruminants (Luong et al., 2000). We previously observed a marked increase in mammary *ACSS2* mRNA abundance at the onset and during lactation (Bionaz and Lor, 2008b), and its transcript pattern corresponded with bovine mammary acetyl-CoA production throughout lactation (Mellenberger et al., 1973).

The expression of classical lipogenic genes in SUBQ increased in response to milk fat depression induced by short-term exogenous *trans*-10,*cis*-12–18:2 (Harvatine et al., 2009). Infusion of CLA led to greater mRNA of *LPL*, *FASN*, and *SCD* as well as the transcription regulators *SREBF1*, *THRSP*, and *PPARG* (Harvatine et al., 2009). We found that some of these genes were also greater statistically (*LPL*, *SCD*) or numerically (*FASN*, *THRSP*) by feeding FSO (Figure 4). This response, along with greater ($P \leq 0.07$) *ADEF* and *LPIN1* (Figure 4), both of which would allow for TAG (Reue and Zhang, 2008) and lipid droplet formation (McManaman et al., 2007), are suggestive of greater lipid deposition. Volume of SUBQ adipocytes increased beyond 120 DIM in cows fed control or lipid-supplemented diets (McNamara et al., 1995). Further, the capacity for esterification at >120 DIM is also expected to be greater in cows fed supplemental lipid. Thus, it would appear that the total pool size of long-chain fatty acids available to SUBQ is a likely driving force for greater lipid filling. However, it remains to be determined if very long chain polyunsaturated fatty acids (PUFA)

Figure 3. Average NE_L intake, milk NE_L yield, and surplus NE_L intake due to feeding ($n = 3$ /diet) a control diet, a milk fat-depressing diet containing fish oil (10 g/kg) and soybean oil (25 g/kg) (FSO), or a diet containing a saturated lipid supplement (EB100, 35 g/kg of DM; Energy Booster, Milk Specialties, Dundee, IL). Asterisks and letters denote treatment $\times$ day interactions ($P < 0.05$).

from fish oil were directly responsible for the changes in gene expression observed. Previous work from our laboratory showed that 20:5 versus 16:0 reduced markedly the mRNA of *LPL*, *SCD*, and *SREBF1* in MacT cells (Kadegowda et al., 2009b), all of which would be required for TAG synthesis. Palmitate induced intracellular lipid droplet formation but 20:5 did not. Further studies with isolated adipocytes would have to be conducted to clarify the tissue's response to specific fatty acids during lactation.

Contrary to a previous short-term (4 d) study of CLA-induced milk fat depression (Harvatine et al., 2009), we did not observe greater *SREBF1* in adipose tissue from cows fed FSO despite lower milk fat production (Figure 4). This was not unexpected because *SREBF1* is acutely regulated by insulin and activates the lipogenic transcriptional cascade primarily in rodent liver (Horton et al., 2002). Furthermore, at least in rodents, *SREBF1* is a well-established *PPARG* target gene (Kast-Woelbern et al., 2004) and we have found evidence in vitro (Kadegowda et al., 2009b) and in vivo (Grauagnard et al., 2009) of the same in cattle. Our temporal data from longissimus muscle of rapidly growing cattle clearly showed that *PPARG* upregulation preceded any increases in *SREBF1*, and *PPARG* responses were paralleled by a battery of genes required for TAG synthesis (Grauagnard et al., 2009).

There is no evidence, to our knowledge, that long-chain fatty acids directly bind to or promote maturation of *SREBF1* to elicit a change in its mRNA expression; that is, most of the negative effects of fatty acids (primarily by PUFA) on *SREBF1* mRNA abundance or transcription activation are via increases in decay or decreases in stability of the mRNA (Postic et al., 2007). Induction of *SREBF1* in liver activates expression of ATP citrate lyase, glucose-6-phosphate dehydrogenase, *ACACA*, *FASN*, *SCD*, and glycerol-3-phosphate acyl-transferase, mitochondrial (*GPAM*) leading to synthesis of palmitic acid, oleic acid, and formation of TAG. Although *SREBF1* activation is associated with greater lipogenic gene expression in rodent liver and adipose tissue (Horton et al., 2003; Li et al., 2003), recent data (Sekiya et al., 2007) found that lipogenic gene expression in adipose was independent of *SREBF1*. Thus, it is doubtful that *SREBF1* is essential for regulation of adipocyte lipogenic/adipogenic gene expression in SUBQ of lactating cows as we have proposed recently in rapidly growing cattle (Grauagnard et al., 2009).

It is noteworthy that cows fed FSO also had numerically greater insulin-induced gene 1 (*INSIG1*) mRNA expression (Figure 4). In murine adipose tissue *INSIG1* mRNA is induced by activation of *PPAR* γ but it occurs relatively late in the adipogenic program, preceded by peak of *PPARG* and *SREBF1* mRNA expression

(Kast-Woelbern et al., 2004). Interestingly, the primary function of *INSIG1* in adipose tissue or liver is to block processing of *SREBF1* (Li et al., 2003), and upregulation of *INSIG1* transcription in adipocytes effectively downregulated expression of *PPARG* and *SREBF1*. The net result of *INSIG1* action is the control of preadipocyte differentiation (Li et al., 2003), thus providing a feedback signaling mechanism to restrict both lipogenesis and adipogenesis. Although limited by number of animals, our data do not seem to support such a mechanism in lactating cow SUBQ because *INSIG1* mRNA

expression was numerically greater in cows fed FSO, which also had greater mRNA of *LPL*, *SCD*, *ADFP*, and *LPIN1*. Greater *INSIG1* expression together with its apparent regulation via $PPAR\gamma$ suggest that the protein encoded by this gene might be pro-lipogenic rather than anti-lipogenic in cattle (Graugnard et al., 2009) as inferred in the mouse (Kast-Woelbern et al., 2004).

Expression of *FABP3* mRNA was lower in cows fed the saturated lipid diet (EB100) compared with control or FSO (Figure 4). Fatty acid binding protein and acyl-

Figure 4. Subcutaneous adipose tissue mRNA abundance after 21 d of feeding ($n = 3/\text{diet}$) a control diet, a milk fat-depressing diet containing fish oil (10 g/kg) and soybean oil (25 g/kg) (FSO), or a diet containing a saturated lipid supplement (EB100, 35 g/kg of DM; Energy Booster, Milk Specialties, Dundee, IL). Letters denote differences ($P \leq 0.07$) and asterisks denote tendencies ($P = 0.13$, *THRSP*) for treatment effects. Gene names are as in Table 1.

CoA binding protein (ACBP or DBI) are the main intracellular fatty acid transporters in nonruminant cells (McArthur et al., 1999). The former has high affinity for free long-chain fatty acids but can also bind acyl-CoA (Whetstone et al., 1986; Frolov et al., 1997). We previously observed the presence of mRNA of all *FABP* isoforms except *FABP2* mRNA in bovine mammary tissue, with greater abundance and upregulation of *FABP3* mRNA during lactation (Bionaz and Loor, 2008a). In a subsequent study, we showed that *FABP3* was the second most abundant transcript (~16%) in mammary tissue among 45 measured, in accordance with the large cytosolic content of its protein in mammary epithelium (Whetstone et al., 1986). However, nonruminant data (Rosen and MacDougald, 2006) have revealed that *FABP4* (another key *PPARG* target gene) is the most abundant and biologically relevant FABP in adipose tissue and our previous work with cattle longissimus muscle tissue provides evidence of the same in cattle. Thus, the biological role of lower *FABP3* due to EB100 in the present study is not readily apparent. Cows with milk fat depression during a short-term *trans*-10,*cis*-12–18:2 abomasal infusion had greater *FABP4* in SUBQ (Harvatine et al., 2009), suggesting that along with lipogenic genes (*FASN*, *THRSP*, *LPL*) it helps coordinate adipogenesis. The lower *FABP3* mRNA due to EB100 could be related to the fact that these cows were diverting dietary fatty acids toward the mammary gland for milk fat synthesis (Table 2, Figure 2).

CONCLUSIONS

We provided evidence that long-term diet-induced milk fat depression and the ensuing increase in surplus intake energy were associated with upregulation of several genes comprising a lipogenic/adipogenic network in subcutaneous adipose tissue. Overall, these changes in gene expression are generally consistent with increased lipid deposition in subcutaneous adipose tissue.

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