Peroxisome proliferator-activated receptor- γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents

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ABSTRACT

Several long-chain fatty acids (LCFA) are natural ligands of nonruminant peroxisome proliferator-activated receptor- γ (PPARG), which, along with its lipogenic target genes, is upregulated in bovine mammary tissue during lactation. Thus, PPARG might represent an important control point of bovine milk fat synthesis. We tested lipogenic gene network expression via quantitative PCR of 19 genes in bovine mammary epithelial cells cultured with 16:0, 18:0, *cis*-9 18:1, *trans*-10 18:1, *trans*-10,*cis*-12 18:2 [*t10c12* conjugated linoleic acid (CLA)], 20:5, ethanol (control), and the PPARG agonist rosiglitazone (ROSI). Triplicate cultures were maintained for 12 h with 50 μ M ROSI or 100 μ M LCFA. Responses common to 16:0 and 18:0 relative to the control included significantly greater expression of *INSIG1* (+298%, +92%), *AGPAT6* (+137%, +169%), *FABP3* (+755%, +338%), and *FABP4* (+171%, 157%). These were coupled with greater intracellular lipid droplet formation and mRNA of *ACSS2*, *LPIN1*, *SCD*, and *SREBF2* in response to 16:0, and greater *DGAT1* and *THRSP* with 18:0. *Trans*-10 18:1 and *t10c12* CLA reduced expression of *FASN* (−60%, −31%), *SCD* (−100%, −357%), and *SREBF1* (−49%, −189%). Furthermore, *t10c12* CLA downregulated *ACSS2*, *FABP3*, *INSIG1*, *SREBF2*, and *THRSP* expression. Expression of *SREBF1* was lower with *cis*-9 18:1 (−140%) and 20:5 (−125%) compared with the control. This latter LCFA also decreased *SCD*, *SREBF2*, and *LPL* expression. No effects of LCFA or ROSI on *PPARG* were observed, but ROSI upregulated (+39% to +269%) expression of *ACACA*, *FASN*, *LPIN1*, *AGPAT6*, *DGAT1*, *SREBF1*, *SREBF2*, and *INSIG1*. Thus, these genes are putative PPARG target genes in bovine mammary cells. This is the first report showing a direct effect of *trans*-10 18:1

on bovine mammary cell lipogenic gene expression. The coordinated upregulation of lipogenic gene networks in response to ROSI and saturated LCFA offers support for PPARG activation in regulating bovine milk fat synthesis.

Key words: nuclear receptor, milk fat, genomics

INTRODUCTION

Long-chain fatty acids (LCFA) alter de novo synthesis of short- and medium-chain fatty acids (Hansen and Knudsen, 1987) as well as mRNA expression (Bauman et al., 2008) in bovine mammary cells. Sterol regulatory element-binding transcription factor 1 (*SREBF1*) and thyroid hormone responsive (SPOT14 homolog, rat; *THRSP*) have been recently linked as key components of *trans(t)*-10,*cis(c)*-12 18:2 [*t10c12* conjugated linoleic acid (CLA)] regulation of milk fat synthesis (Harvatine and Bauman, 2006). However, those are most likely indirect effects because fatty acids cannot bind directly to these transcriptional regulators (Pegorier et al., 2004).

In nonruminants, most LCFA, and specifically polyunsaturated fatty acids (PUFA), are natural ligands and bind to peroxisome proliferator-activated receptor- γ (PPARG), eliciting changes in gene expression and rates of lipogenesis (Bensinger and Tontonoz, 2008; Berger and Moller, 2002; Desvergne et al., 2006). Messenger RNA expression of PPARG and several potential target genes in mammary tissue was upregulated (Bionaz and Loor, 2008b) at the onset and throughout lactation in dairy cows. Therefore, it was proposed that milk fat synthesis regulation via exogenous or mammary-synthesized LCFA ligands (e.g., palmitic acid) could take place through PPARG-mediated changes in gene expression.

There is a high correlation between mRNA expression of target genes and recruitment of lipogenic transcription factors and their coregulatory proteins to promoter regions, suggesting that gene expression analysis is useful for inferring transcriptional activity (Bennett et al., 2008). In addition, an increase in

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metabolic flux requires overexpression of most of the enzymes in a biosynthetic pathway, challenging the idea of a “limiting enzyme” (Morandini et al., 2005). This is an important consideration when interpreting data from transcriptomics studies. Therefore, measurement of mRNA for multiple genes and their networks in a pathway is essential.

Our central hypothesis was that activation of PPARG in bovine mammary cells would upregulate mRNA expression of lipogenic target genes identified primarily in rodent studies. Furthermore, we hypothesized that different LCFA regulate expression of genes controlling networks of lipid metabolism to various extents, and potentially through PPARG. The specific objectives were to determine the effects of PPARG activation as well as exogenous saturated and unsaturated LCFA on mRNA expression of genes that compose an interactive network controlling in vivo bovine mammary cell lipid metabolism (Bionaz and Looor, 2008a,b).

MATERIALS AND METHODS

Cell Culture and Treatments

Bovine mammary epithelial (MacT; provided by Daniel Peterson, California Polytechnic State University, San Luis Obispo) cells were allowed to grow in canted-neck, vented, 75-cm² flasks (430641, Corning, Glendale, AZ) in HyQ RPMI-1640 media (HyClone, Logan, UT). Cultures were maintained in a water-jacketed incubator (model 3158, Forma Scientific, Marietta, OH) with 5% CO₂ in air at 37°C. Culture medium was changed every 24 h and cells were subcultured to 70 to 80% confluence (approximately every 2 to 3 d) by rinsing once with 10 mL of PBS buffer without Ca and Mg (sh3002802, HyClone), 3 mL of 0.25% trypsin (sh3004201, Hyclone) plus 3 mL of Cellstripper (25-056-Cl, Cellgro, Herndon, VA), and incubated at 37°C for 5 to 10 min (i.e., until evidence of cell detachment). Trypsin activity was inhibited by addition of 6 mL of fresh culture media at 37°C.

Approximately 48 h prior to the last subculture before initializing the experiment, cells were allowed to grow in a basal medium similar to that of Peterson et al. (2004), with modifications. The basal medium was composed of Minimum Essential Medium/Earle's Balanced Salts HyQ (MEM/EBSS, HyClone) with insulin (5 mg/L, I6634, Sigma, St. Louis, MO), hydrocortisone (1 mg/L, H0888, Sigma), transferrin (5 mg/L, T1428, Sigma), ascorbic acid (5 µM/L, A4544, Sigma), sodium acetate (5 mM/L, S5636, Sigma), and penicillin/streptomycin (10 mL/L, sv30010, HyClone). The basal medium was supplemented with fetal bovine serum (10%, Gibco, Invitrogen, Carlsbad, CA) and

growth-promoting hormones (1 mg/L of progesterone, P8783, Sigma; 0.05% lactalbumin, L5385, Sigma; 0.05% α-lactose, 47287-U, Sigma). At approximately 24 h before applying treatments (approximately 90% confluence), cells were cultured in a lactogenic medium as reported by Peterson et al. (2004), with modifications. The lactogenic medium was prepared as the basal medium, except that high-glucose Dulbecco's modified Eagle's medium (HG-DMEM, HyClone) was used. The lactogenic medium was devoid of fetal bovine serum and was supplemented with BSA (1 g/L) and prolactin (2.5 mg/L).

Treatments were the known PPARG ligands (Walkey and Spiegelman, 2008) rosiglitazone (**ROSI**; Cayman Chemical, Ann Harbor, MI), stearate (18:0; N-18-A, Nu-Chek Prep Inc., Elysian, MN), and oleate (*cis*-9 18:1, 1022, Matreya, Pleasant Gap, PA) as well as eicosapentanoate (20:5, N-20-A, Nu-Chek Prep Inc.), palmitate (16:0; N-16-A, Nu-Chek Prep Inc.), *trans*-10 18:1 (kindly provided by M. Pete Yurawecz, US Food and Drug Administration, Washington, DC), and *tl0c12* CLA (no. 1249, Matreya).

Preparation of LCFA and ROSI

The stock solution of 30 mM LCFA was prepared in 13 × 100 mm Pyrex glass tubes with screw caps by saponification at 42°C with an equimolar solution of NaOH in water. After saponification, the soap was suspended with absolute ethanol to obtain a final solution of 95% ethanol. After preparation, the LCFA stock solution was screw-capped using paraffin and stored at −20°C until use. A 95% ethanol + 30 mM NaOH solution served as the control. Most of these LCFA are PPARG ligands in rodents as well as in human adipocyte cultures (Sauma et al., 2006), and exogenous *tl0c12* CLA and 20:5 have been shown to elicit different responses in bovine mammary lipid synthesis (Looor et al., 2005b; Harvatine and Bauman, 2006).

Preliminary Time-Course Study. The MacT cells from several 75-cm² vented flasks were pooled after trypsinization in a 50-mL tube and mixed thoroughly before transfer to 6-well plates for addition of treatments. As reported above, cultures were maintained for 24 h in a lactogenic medium before treatments were applied (approximately 70% confluence). For RNA extraction, cells were cultured in triplicate with 100 µM palmitate (16:0), *trans*-10, *cis*-12 18:2 (*tl0c12* CLA), and 50 µM ROSI plus the control for 0, 3, 6, 12, and 24 h in a lactogenic medium. Cells were harvested in 1 mL of TRIzol reagent (Invitrogen) and stored at −80°C until RNA extraction.

The preliminary study was conducted to determine a suitable incubation time based on peak mRNA ex-

pression of the lipogenic genes acetyl-coenzyme A carboxylase (*ACACA*), fatty acid synthetase (*FASN*), stearoyl-CoA desaturase (*SCD*), *SREBF1*, lipin 1 (*LPIN1*), and the fatty acid translocase CD36 molecule [thrombospondin receptor] (*CD36*; Figure 1). The PCR data were calculated using a 6-point standard curve and normalized by the geometric mean of *MRPL39*, *UXT*, and *RPS9*. Final data were obtained as the percentage change relative to time 0 for all treatments and were corrected by the control data. Statistical analysis of the qPCR and Oil Red O staining results was performed using the MIXED procedure (version 9.1, SAS Institute, Cary, NC) with repeated measures to evaluate the effects of treatment, time, and treatment $\times$ time on normalized mRNA abundance of genes. The model included the fixed effect of time (0, 3, 6, 12, and 24 h) and the random effect of culture plates. For intracytoplasmic lipid droplets, measurement cells were subjected to the same conditions as reported for RNA extraction in duplicate for 24 h, and lipid droplets were visualized with Oil Red O staining (see below). The model included 0 h as a covariate with treatments as fixed effects and culture plates as the random effect. Results indicated that a 12-h incubation was suitable to obtain maximal responses in mRNA expression. Clearly, this represented a compromise because it is unlikely that all genes would have maximal expression at the same point in time.

Final Experimental Setup. Subculture was performed several times to obtain large numbers of cells to initiate the main experiment. When the numbers of cells were deemed adequate to initiate the experiment, all the cells were pooled in a 50-mL sterile tube with fresh medium at 37°C and mixed thoroughly before plating in new 75-cm² flasks with lactogenic medium. This was done to obtain the same initial number of cells in each flask, and thus to have high consistency in initial conditions between samples. Before initiating the experiment, cells remained in the lactogenic medium (changed every 24 h) for approximately 2 d, when they reached $\geq 90\%$ confluence, and the treatments were applied.

Cells were cultured with 100 μM each LCFA or 50 μM ROSI (Walkey and Spiegelman, 2008). Each treatment was performed in triplicate (i.e., 3 separate flasks per treatment) and cells were harvested at 12 h of incubation.

RNA Extraction, RNA Quality Assessment, and Real-Time PCR

Total RNA was extracted from the cells using ice-cold TRIzol reagent (Lloor et al., 2005a). Concentrations of

RNA were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genomic DNA was removed with DNase using RNeasy Mini Kit columns (Qiagen, Valencia, CA). A portion of the RNA was diluted to 100 mg/L using DNase/RNase-free water before quantitative real-time PCR (qPCR). Sufficient cDNA was prepared to run all selected genes. Each cDNA was synthesized by qPCR using 100 ng of RNA, 1 μL of dT18 (Operon Biotechnologies, Huntsville, AL), 1 μL of 10 mM deoxynucleotide 5'-triphosphate mix (Invitrogen), 1 μL of Random Primers (Invitrogen), and 7 μL of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min in an Eppendorf Mastercycler Gradient and kept on ice for 3 min. A total of 9 μL of Master Mix, composed of 4.5 μL of 5 $\times$ First-Strand Buffer, 1 μL of 0.1 M dithiothreitol, 0.25 μL (100 U) of SuperScript III RT (Invitrogen), 0.25 μL of RNase Inhibitor (Promega, Madison, WI), and 3 μL of DNase/RNase-free water was added. The reaction was performed using the following temperature program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. Complementary DNA was then diluted 1:3 with DNase/RNase-free water.

For qPCR, 4 μL of diluted cDNA was combined with 6 μL of a mixture composed of 5 μL of 1 $\times$ SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL of DNase/RNase-free water in a MicroAmp Optical 384-well reaction plate (Applied Biosystems). Each sample was run in triplicate and a 6-point relative standard curve (4-fold dilution) plus the nontemplate control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. 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 analyzed with the 7900 HT Sequence Detection Systems Software (version 2.2.3, Applied Biosystems).

Primer Design and Testing

Primer Express 3.0 software (Applied Biosystems), optimized for use with Applied Biosystems qPCR systems, was used for primer design, using the default features except for the amplicon length, which was fixed at a minimum of 100 bp. Primers were designed to span exon-exon boundaries when possible to avoid amplification of genomic DNA. The exon junctions were uncovered by blasting the sequence against bovine genome (Genome Browser Gateway, 2008). Primers were aligned against publicly available sequences in NCBI

Figure 1. Preliminary study to examine temporal mRNA expression patterns (percentage relative to 0 h) of lipogenic genes (*ACACA*, *FASN*, *SCD*, *SREBF1*, *LPIN1*) and a fatty acid translocase (*CD36*) in response to palmitate (16:0), *trans*-10,*cis*-12 18:2 (*t10c12* CLA), or rosiglitazone (ROSI) after 0, 3, 6, 12, or 24 h of incubation. Data were corrected by the control at each time point. *ROSI, #*t10c12* CLA, and \$16:0 denote significant ($P < 0.05$) differences relative to 0 h. Letters (a,b,c) denote significant differences among treatments at the specific time point. Time, treatment, and treatment $\times$ time were significant ($P \leq 0.05$) for all genes except for *ACACA*, with a time effect ($P = 0.06$). *ACACA* = acetyl-coenzyme A carboxylase; *FASN* = fatty acid synthetase; *SCD* = stearoyl-coenzyme A desaturase; *SREBF1* = sterol regulatory element-binding transcription factor 1, *LPIN1* = lipin 1; *CD36* = the fatty acid translocase CD36 molecule [thrombospondin receptor].

(National Center of Biotechnology Information, 2008) and UCSC Genome Browser (Genome Browser Gateway, 2008). Before qPCR, primers were tested using the same protocol as for qPCR but without the dissociation step in a 20- μ L reaction. Five microliters of the PCR product was run in a 2% agarose gel stained with ethidium bromide to assess the presence of the product

to a expected size and the presence of primer-dimer; the rest was purified using a Qiaquick PCR purification kit (Qiagen) and sent to be sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. Only primers with high specificity evaluated by a single band on agarose gel, the absence of primer-dimer, amplification

of the right cDNA as verified by sequencing, and a unique peak in the dissociation curve after the qPCR reaction were used. Sequencing results for all genes are reported in a previous publication (Bionaz and Loor, 2008b).

Additional details on selected genes and real-time PCR performance for each gene analyzed are reported in Supplemental Tables 1 and 2 (<http://jds.fass.org/content/vol92/issue9>).

Oil Red O Staining

The intracytoplasmic lipid droplets in cells were visualized with Oil Red O staining, which was applied to cells fixed with 10% formalin after a wash with PBS (Carson, 1990). Fixation was performed for 30 to 60 min. Fixed cells were washed gently with water, and 2 mL of 60% isopropanol was added to cover the bottom of each well and kept for 5 min. Isopropanol was removed and 1 mL of a filtered working solution of Oil Red O stain (300 mg/100 mL of 99% isopropanol) was added. Cells were incubated for 5 min, Oil Red O staining was removed, and cells were rinsed with tap water. Then, 1 mL of hematoxylin (HHS-16, Sigma) was added for 1 min to stain the nucleus of the cells, and cells were rinsed carefully with tap water. Pictures were taken using an inverted microscope at 400 $\times$ magnification (Supplemental Figures 1 and 2; <http://jds.fass.org/content/vol92/issue9>). Quantification of intracytoplasmic triglycerides was determined by extracting Oil Red O staining as described previously (Ramirez-Zacarias et al., 1992). The quantification of the extract was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) at 510 nm. A 6-point standard curve with 2-fold dilution of Oil Red O solution was used to obtain the final values (Figure 2).

Data Transformation, Relative mRNA Abundance, and Statistical Analysis

Raw qPCR data were transformed based on a 6-point standard curve and normalized by the geometric mean of 3 previously tested internal control genes (*MRPL39*, *RPS9*, and *UXT*; Bionaz and Loor, 2007). Normalized data were further transformed to obtain a perfect mean of 0.0% for the control, and differences in mRNA expression of treatments were transformed to percentage of mRNA relative to the control mean (Bionaz and Loor, 2008a,b). The median of the relative percentages of mRNA abundance among genes was calculated as reported previously (Bionaz and Loor, 2008b); that is, $1/E^{(\Delta C_t)}$, where E is the efficiency of PCR amplifica-

Figure 2. Spectrophotometric quantification of Oil Red O staining from data reported in Supplemental Figure 2 (<http://jds.fass.org/content/vol92/issue9>). An asterisk (*) denotes significant ($P < 0.05$) differences from the control. CTR = media control (without ethanol); CTROH = ethanol control or true control; *t* = *trans*; CLA = conjugated linoleic acid; ROSI = rosiglitazone.

tion and ΔC_t is calculated as [C_t gene – geometrical mean of C_t internal controls] considering all treatments (see Supplemental Table 2 for PCR performance and Supplemental Figure 3 for relative percentage mRNA abundance among measured genes; <http://jds.fass.org/content/vol92/issue9>). The GLM procedure (version 9.1, SAS Institute Inc., Cary, NC) was used to evaluate the treatment effects on percentage of mRNA abundance relative to the control and lipid droplet formation. Fixed effects in the model were treatments, whereas the random effects were replicates ($n = 3$ cultures/treatment). Treatment means were separated using Fisher's least significant difference pair-wise comparisons (Peterson et al., 2004). Significance was declared at $P \leq 0.05$.

Expression Clustering and Gene Network Development

Hierarchical clustering (Figure 3) was performed using fold changes in mRNA expression for each treatment relative to the control by using Genesis software (Sturn et al., 2002). Summary networks among the genes most affected by treatments were developed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA; www.ingenuity.com; Figure 4; Supplemental Figures 4 to 6; <http://jds.fass.org/content/vol92/issue9>). The gene expression data set comprising gene identifiers, corresponding expression values, and pair-wise comparison P -values were uploaded into IPA. Connections among genes were developed based on results from this study as well as on known relationships available in the IPA knowledge base. This is a proprietary manually curated database containing relationships from the published literature on rodents and humans.

RESULTS

Lipid Droplet Formation

Compared with the control, cells cultured with 16:0, 18:0, or *t*10*c*12 CLA had significant increases of 140, 80, and 250%, respectively, of intracellular TAG (Figure 2; Supplemental Figures 1 and 2). Increased TAG accumulation in MacT has been reported previously for 16:0, 18:0, and *cis*-9 18:1 (Yonezawa et al., 2008). Others have reported increased intracellular content of 18:0, *cis*-9 18:1, and *trans*-11 18:1 in MacT cells when these LCFA were supplemented (0, 50, or 100 μ M) in culture medium (Jayan and Herbein, 2000).

mRNA Expression of Genes Associated with LCFA Uptake, Intracellular Activation, Intracellular Transport, De Novo Synthesis, and Esterification

Expression of lipoprotein lipase (*LPL*) was significantly lower with *cis*-9 18:1 (−150%), *trans*-10 18:1 (−252%), and 20:5 (−118%) relative to the control (Table 1). In contrast, expression of *CD36* was significantly greater with 18:0 (1,009%), 20:5 (1,043%), and *t*10*c*12 CLA (953%).

Relative to the control, mRNA expression of acyl-CoA synthetase short-chain family member 2 (*ACSS2*) was upregulated only with 16:0 (168%), but expression of acyl-CoA synthetase long-chain family member 1 (*ACSL1*) was not affected by treatments. The *ACSS2* was markedly downregulated (−31%) by *t*10*c*12 CLA compared with the control (Table 1). The mRNA abundance of fatty acid-binding protein 3, muscle and heart (mammary-derived growth inhibitor; *FABP3*), and fatty acid-binding protein 4 (*FABP4*) was greater with both 16:0 (755% and 171%) and 18:0 (338% and 157%). Oleic acid also resulted in greater expression of *FABP4* (69%). In contrast, *t*10*c*12 CLA (−169%) and *cis*-9 18:1 (−172%) decreased *FABP3* expression.

Among genes associated with de novo fatty acid synthesis, expression of *ACACA* and *FASN* was lower with 18:0 (−56 and −69%) and *t*10*c*12 CLA (−97 and −31%) relative to the control (Table 1). *Trans*-10 18:1 decreased expression of *FASN* by 61%. The mRNA expression of *SCD* was upregulated with 16:0 (95%) but was downregulated by the presence of LCFA containing 1 or more double bonds, namely, *cis*-9 18:1 (−428%), *trans*-10 18:1 (−100%), *t*10*c*12 CLA (−357%), and 20:5 (−205%; Table 1). Surprisingly, 18:0 did not affect *SCD* mRNA expression.

Although none of the treatments affected expression of glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*), expression of diacylglycerol *O*-acyltrans-

Figure 3. Hierarchical clustering of gene expression data for each treatment relative to the control using Genesis software (Sturn et al., 2002). Dendrograms allow visualization of clusters of similarity in expression pattern among genes between treatments (links denoted by the lines at the left side of the picture), among treatments between genes (denoted by the lines at the top of the figure), or both. Cutoffs for expression level are denoted by shades of gray and black according to the intensity bar at the top of the figure. Gray intensity denotes an increase (toward black, 1- to 3-fold upregulation vs. control) or decrease (toward light gray, 1- to 3-fold downregulation) in expression relative to the control (refer to Table 1 for statistical differences). White dots denote the largest responses (up or down) in mRNA expression for each gene. *t* = *trans*; *c* = *cis*; CLA = conjugated linoleic acid; ROSI = rosiglitazone; *CD36* = the fatty acid translocase CD36 molecule [thrombospondin receptor]; *DGAT1* = diacylglycerol *O*-acyltransferase homolog 1; *SCAP* = SREBF chaperone; *AGPAT6* = 1-acylglycerol-3-phosphate *O*-acyltransferase 6; *FASN* = fatty acid synthetase; *ACACA* = acetyl-coenzyme A carboxylase; *SREBF1* = sterol regulatory element-binding transcription factor 1; *LPL* = lipoprotein lipase; *FABP4* = fatty acid-binding protein 4; *ACSS2* = acyl-coenzyme A synthetase short-chain family member 2; *FABP3* = fatty acid-binding protein 3; *LPIN1* = lipin 1; *INSIG1* = insulin-induced gene 1; *SCD* = stearoyl-coenzyme A desaturase; *SREBF2* = sterol regulatory element-binding transcription factor 2; *THRSP* = thyroid hormone responsive (SPOT14 homolog).

ferase homolog 1 (mouse; *DGAT1*) was greater with 18:0 (89%). Expression of 1-acylglycerol-3-phosphate *O*-acyltransferase 6 (*AGPAT6*) was greater in response

Figure 4. Summary of potential regulatory networks among genes studied in response to peroxisome proliferator-activated receptor- γ (PPARG) activation via rosiglitazone or palmitate (16:0). Networks were developed with Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA; www.ingenuity.com) using data from the present study (Table 1) and the IPA knowledge base, including known relationships among genes at the level of mRNA expression (E), protein-DNA (PD), transcription (T), and protein-protein (PP). Genes with a black background were upregulated by the specific treatment. Genes with a gray background were not affected significantly by treatments. Edges denote genes whose transcription is under the control of PPARG, SREBF1, LPIN1, SREBF2, or their combination. The responses with rosiglitazone (ROSI) provide evidence of novel PPARG target genes (e.g., LPIN1, SREBF1, SREBF2, INSIG1), all of which could coordinate lipid synthesis in mammary epithelial cells. Data with 16:0 highlight the possibility that this long-chain fatty acid could regulate mRNA expression of FABP3, FABP4, LPIN1, SCD, and INSIG1 through direct binding to PPARG. It also is possible that 16:0 could bind to unidentified transcription regulators (TF) or nuclear receptors and elicit a response in target-gene mRNA expression. LPIN1 = lipin 1; THRSP = thyroid hormone responsive (SPOT14 homolog); SREBF2 = sterol regulatory element-binding transcription factor 2; INSIG1 = insulin-induced gene 1; SCAP = SREBF chaperone; SREBF1 = sterol regulatory element-binding transcription factor 1; DGAT1 = diacylglycerol *O*-acyltransferase homolog 1; AGPAT6 = 1-acylglycerol-3-phosphate *O*-acyltransferase 6; GPAM = glycerol-3-phosphate acyltransferase, mitochondrial; SCD = stearoyl-coenzyme A desaturase; FASN = fatty acid synthetase; ACACA = acetyl-coenzyme A carboxylase; ACSS2 = acyl-coenzyme A synthetase short-chain family member 2; ACSL1 = acyl-coenzyme A synthetase long-chain family member 1; CD36 = the fatty acid translocase CD36 molecule [thrombospondin receptor]; FABP3 = fatty acid-binding protein 3; LPL = lipoprotein lipase; FABP4 = fatty acid-binding protein 4.

to all LCFA treatments except *trans*-10 18:1. Expression of lipin 1 (*LPIN1*) was greater with 16:0 (141%) but was lower with *cis*-9 18:1 (−63%).

The PPARG agonist ROSI did not affect expression of genes associated with LCFA uptake or intracellular activation and transport, including *LPL* (Table 1). However, it resulted in greater expression of *ACACA* (85%), *AGPAT6* (269%), *DGAT1* (76%), *FASN* (39%), and *LPIN1* (116%), suggesting that these genes are putative PPARG target genes in MacT cells and likely in bovine mammary tissue.

mRNA Expression of Transcriptional Regulators

Relative to the control, *SREBF1* expression was lower with *cis*-9 18:1 (−140%), *trans*-10 18:1 (−49%), *t10c12* CLA (−189%), and 20:5 (−125%; Table 1). Similarly, sterol regulatory element-binding transcription factor 2 (*SREBF2*) expression was lower with *cis*-9 18:1 (−83%), *t10c12* CLA (−71%), and 20:5 (−35%) but was greater with 16:0 (53%). Among the genes known to regulate *SREBP* activity, mRNA expression of *SREBF* chaperone (*SCAP*) was lower (−76%) with 20:5. Expression of insulin-induced gene 1 (*INSIG1*) was greater with 16:0 (298%), 18:0 (92%), and 20:5 (84%) but was lower with *cis*-9 18:1 (−93%) and *t10c12* CLA (−95%). Expression of the transcription factor *THRSP* was greater with 18:0 (97%) and lower with *t10c12* CLA (−73%) relative to the control. None of the treatments affected *PPARG* expression. Rosiglitazone led to greater expression of *SREBF1* (100%), *SREBF2* (35%), and *INSIG1* (142%), also suggesting that these genes are putative PPARG target genes in bovine.

Gene Expression Clusters

The hierarchical algorithm implemented by Genesis attempts to find successive clusters using previously established clusters; that is, it uses a “top-down” (or divisive) approach, beginning with the whole set, and proceeds to divide it into successively smaller clusters (Sturn et al., 2002). This clustering analysis revealed that the mRNA expression profiles obtained essentially grouped into 3 patterns of expression (Figure 3). Treatments 16:0, 18:0, and ROSI were grouped in a cluster characterized primarily by upregulation (Table 1) of several genes, including *FABP4*, *ACSS2*, *FABP3*, *LPIN1*, *INSIG1*, *THRSP*, and *DGAT1*. Within this group, the most similar expression pattern across the 19 genes examined was that between 16:0 and 18:0. A second cluster was composed of expression patterns obtained with treatments *trans*-10 18:1, *t10c12* CLA, and *cis*-9 18:1. Within this cluster, expressions patterns with *t10c12* CLA and *cis*-9 18:1 were further grouped

together based on their more similar expression relative to *trans*-10 18:1. The third major cluster of expression was obtained with treatment 20:5.

DISCUSSION

PPARG Regulation of Mammary Cell Lipogenic Gene Networks

The coordinated suppression of a small subset of lipogenic genes (e.g., *ACACA*, *FASN*, *SCD*) during milk fat depression led to the inference that milk fat synthesis regulation could be controlled at a common regulatory point (Peterson et al., 2003, 2004). Studies have largely focused on the putative role of *SREBF1* and, more recently, on the transcription factor *THRSP* (Harvatine and Bauman, 2006). In addition to *SREBF1*, *SREBF2* can induce mRNA expression of genes involved in fatty acid synthesis besides its role in cholesterol synthesis (Horton et al., 2003). Previous data, however, are suggestive of a marginal function for *THRSP* in bovine mammary tissue during the normal course of lactation (Bionaz and Loor, 2008b). The above-mentioned transcription regulators could independently exert some level of control over milk fat synthesis, but it is unlikely that LCFA interact with them directly to elicit a response; that is, effects are probably indirect and likely dependent on other upstream transcription regulators able to bind LCFA or on effects on other enzymes (Pegorier et al., 2004). Gene network analysis in bovine mammary tissue showed that expression of *PPARG* and its putative target genes was upregulated during lactation, suggesting a role for this nuclear receptor in the regulation of milk fat synthesis (Bionaz and Loor, 2008a,b). Peroxisome proliferator-activated receptor- γ is one of the most studied nuclear receptors because of its central role in regulating metabolism in relation to the composition and concentration of LCFA in the cellular environment (Desvergne et al., 2006).

The fact that LCFA are potent agonists of PPARG in nonruminants led us to infer that it could be a potential player in mammary lipid metabolism. Among the genes studied, *CD36*, *LPL*, *FABP4*, and *ACSL1* are known PPARG target genes in nonruminants (Berger and Moller, 2002). In addition, genes related to de novo fatty acid synthesis (*ACACA*, *FASN*), desaturation (*SCD*), and TAG synthesis (*AGPAT6*) are upregulated in adipose tissue of rats (Way et al., 2001) or humans (Kolpak et al., 2007) by PPARG agonist treatment (GW1929, ROSI). As summarized in Figure 4, treatment with ROSI increased the expression of genes related to de novo fatty acid synthesis (*ACACA*, *FASN*), TAG synthesis (*AGPAT6*, *DGAT1*, *LPIN1*), transcriptional regulation of lipid synthesis (*SREBF1*,

Table 1. Effect of long-chain fatty acids (LCFA) and rosiglitazone (ROSI) on percentage of mRNA expression change relative to the control

Gene ¹	Treatment							SEM	P-value
	Control	16:0	18:0	<i>Cis</i> -9 18:1	<i>Trans</i> -10 18:1	<i>tl0c12</i> CLA ²	20:5		
	% change in mRNA expression relative to control								
<i>ACACA</i>	0.0 ^b	−4.6 ^{bc}	−55.5 ^{cd}	−37.0 ^{bc}	−41.4 ^{bc}	−97.0 ^d	−46.5 ^{bcd}	85.1 ^a	0.001
<i>ACSL1</i>	0.0	−6.1	26.7	11.3	−1.3	4.4	17.7	18.4	0.407
<i>ACSS2</i>	0.0 ^{bcd}	168.5 ^a	4.4 ^{bc}	−25.5 ^{de}	−16.1 ^{cde}	−30.4 ^e	4.1 ^{bc}	19.2 ^b	0.001
<i>AGPAT6</i>	0.0 ^d	137.4 ^{bc}	168.5 ^b	159.2 ^b	46.6 ^{cd}	104.1 ^{bc}	190.7 ^{ab}	269.2 ^a	0.001
<i>CD36</i>	0.0 ^c	370.5 ^c	1,008.9 ^a	293.4 ^c	446.4 ^{bc}	953.0 ^{ab}	1,043.0 ^a	77.0 ^c	0.002
<i>DGAT1</i>	0.0 ^{cd}	43.4 ^{abc}	89.3 ^a	44.6 ^{abc}	27.1 ^{bc}	40.9 ^{abc}	−27.2 ^d	76.3 ^{ab}	0.005
<i>FABP3</i>	0.0 ^{cd}	754.7 ^a	338.4 ^b	−171.7 ^e	−92.1 ^{de}	−169.1 ^e	12.2 ^{cd}	39.5 ^c	0.001
<i>FABP4</i>	0.0 ^c	170.9 ^a	157.2 ^a	69.0 ^b	0.4 ^c	0.6 ^c	−3.4 ^c	36.4 ^{bc}	0.001
<i>FASN</i>	0.0 ^b	−1.0 ^{bc}	−69.4 ^e	−8.0 ^{bc}	−60.1 ^{de}	−30.6 ^{cd}	−16.4 ^{bc}	38.5 ^a	0.001
<i>GPAM</i>	0.0	−22.2	48.3	48.8	49.1	71.4	15.6	19.9	0.204
<i>INSIG1</i>	0.0 ^d	298.3 ^a	91.7 ^{bc}	−93.2 ^e	−15.3 ^d	−95.3 ^e	84.2 ^c	142.3 ^b	0.001
<i>LPIN1</i>	0.0 ^{bc}	140.8 ^a	30.0 ^b	−63.4 ^d	−31.5 ^{cd}	−33.9 ^{cd}	−38.5 ^{cd}	117.5 ^a	0.001
<i>LPL</i>	0.0 ^a	−27.8 ^a	−2.7 ^a	−150.1 ^c	−251.9 ^d	−78.6 ^{abc}	−118.2 ^{bc}	−48.4 ^{ab}	0.001
<i>PPARG</i>	0.0	23.4	22.2	−12.9	−13.6	13.7	0.8	8.6	0.560
<i>SCAP</i>	0.0 ^a	−13.9 ^a	−10.0 ^a	17.2 ^a	9.1 ^a	12.6 ^a	−76.1 ^b	31.7 ^a	0.012
<i>SCD</i>	0.0 ^b	94.8 ^a	4.6 ^b	−428.2 ^f	−99.7 ^c	−357.4 ^e	−204.6 ^d	55.3 ^{ab}	0.001
<i>SREBF1</i>	0.0 ^b	5.9 ^b	−5.0 ^b	−139.5 ^d	−48.5 ^c	−188.6 ^e	−125.3 ^d	100.0 ^a	0.001
<i>SREBF2</i>	0.0 ^b	52.9 ^a	2.4 ^b	−82.7 ^d	−18.8 ^{bc}	−71.4 ^d	−34.8 ^c	35.3 ^a	0.001
<i>THRSP</i>	0.0 ^{bc}	9.0 ^{bc}	97.3 ^a	−22.1 ^{cd}	−32.6 ^{cd}	−73.2 ^d	10.0 ^{bc}	44.0 ^{ab}	0.001

^{a–f}Values in the same row with different superscript letters differ significantly from each other ($P < 0.05$).

¹*ACACA* = acetyl-coenzyme A carboxylase; *ACSL1* = acyl-coenzyme A synthetase long-chain family member 1; *ACSS2* = acyl-coenzyme A synthetase short-chain family member 2; *AGPAT6* = 1-acylglycerol-3-phosphate *O*-acyltransferase 6; *CD36* = the fatty acid translocase CD36 molecule [thrombospondin receptor]; *DGAT1* = diacylglycerol *O*-acyltransferase homolog 1; *FABP3* = fatty acid-binding protein 3; *FABP4* = fatty acid-binding protein 4; *FASN* = fatty acid synthetase; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *INSIG1* = insulin-induced gene 1; *LPIN1* = lipin 1; *LPL* = lipoprotein lipase; *PPARG* = peroxisome proliferator-activated receptor- γ ; *SCAP* = SREBF chaperone; *SCD* = stearoyl-coenzyme A desaturase; *SREBF1* = sterol regulatory element-binding transcription factor 1; *SREBF2* = sterol regulatory element-binding transcription factor 2; *THRSP* = thyroid hormone responsive (SPOT14 homolog).

²*tl0c12* CLA = *trans*-10,*cis*-12 18:2; CLA = conjugated linoleic acid.

SREBF2), and the *SREBF1*-repressor *INSIG1*. These observations strongly indicate a role for PPARG in de novo fatty acid synthesis regulation, partly because the estimated control flux coefficient through the pathway was estimated to be 63% for *ACACA* and for 37% for *FASN* in bovine mammary homogenates (Wright et al., 2006). Together, the results support a previous suggestion (Bionaz and Loor, 2008b) that PPARG activation could be a regulatory point of milk fat synthesis.

Our observations with ROSI are striking because they suggest that a single nuclear receptor with LCFA as its natural ligands could potentially regulate not only target genes (*ACACA*, *FASN*, *LPIN1*), as in nonruminants, but also a subset of lipogenic transcription factors (i.e., *SREBF1* and *SREBF2*). In addition to potentially binding up to 2 fatty acids simultaneously, it has been proposed (Itoh et al., 2008) that the large pocket in PPARG could allow for a graded response to varying compositions of the cellular pool of LCFA ligands (e.g., saturated and unsaturated LCFA). A complex regulatory network might be at work in mammary cells such that several proteins serve as putative checkpoints to regulate milk fat synthesis. As an example, increased *SREBF1* expression upon PPARG activation could lead to upregulation of downstream lipogenic genes such as *ACACA*, *FASN*, and *AGPAT6* (Figure 4). Under a different scenario, if the observed increase in *INSIG1* mRNA expression attributable to ROSI extended to the protein level, more *SREBP1* might be retained in the endoplasmic reticulum via *SCAP-INSIG1* binding; that is, *SREBP1* would be rendered inactive (Horton et al., 2003; Kast-Woelbern et al., 2004), as suggested previously (Bionaz and Loor, 2008b).

Rosiglitazone did not affect the expression of *LPL* and *ACSL1*, both known PPARG target genes in non-ruminant adipose tissue, suggesting that regulation of lipogenic gene expression through PPARG might differ between adipose tissue and mammary epithelial cells. Alternatively, there might be species differences in the genes regulated by PPARG as well as in the potency of their regulation. The *LPL* was one of the least abundant transcripts in MacT cells (Supplemental Figure 3). This might be indicative of an adaptation of immortalized mammary cells, which do not require *LPL* because, in vivo, this protein is exported to the endothelium for processing of lipoproteins. We did not observe an increase in lipid droplet formation with ROSI relative to the control despite the greater mRNA expression of lipogenic genes and ample availability of acetate in culture medium, which, coupled with greater *ACACA* and *FASN* mRNA expression, should have provided short-chain fatty acids and 16:0 for lipid droplet formation. We speculate, based on those data, that

mammary cells have a requirement for LCFA for lipid droplet formation.

LCFA Regulation of Mammary Lipogenic Genes Through PPARG

Palmitic Acid. As in other tissues, mammary LCFA uptake and metabolism are likely orchestrated by a large set of proteins (Bionaz and Loor, 2008b). Results with ROSI suggest that several of the examined lipogenic genes are PPARG targets; thus, their level of expression in response to LCFA provides an indication of whether they might act through this nuclear receptor. Clustering analysis (Figure 3) revealed that the similarity of expression patterns among tested genes relative to ROSI was 16:0 > 18:0 > 20:5 > *t10c12 CLA* = *cis-9 18:1* > *trans-10 18:1*. Together, these data suggest that the effect of 16:0 and 18:0 on the transcription of lipogenic genes in MacT cells is partly driven by the activation of PPARG (Figure 4).

Genes upregulated by 16:0 and ROSI included *AGPAT6*, *LPIN1*, *INSIG1*, and *SREBF2* (Table 1 and Figure 4). Furthermore, 16:0 was the only LCFA that concomitantly increased *ACSS2* expression [a gene encoding a cytosolic protein with high specificity for acetate and, to some extent, propionate (Fujino et al., 2001)] as well as intracytoplasmic TAG content (Figure 2 and Supplemental Figures 1 and 2). A role for 16:0 in regulating de novo synthesis was observed previously in bovine and ovine dispersed mammary epithelial cells when exogenous 16:0 led to increased synthesis and incorporation of butyrate into TAG (Hansen and Knudsen, 1987). In addition, 16:0 is the preferred substrate for the initial acylation of L- α -glycerolphosphate via *GPAM*, leading to formation of *sn*-1-lysophosphatidic acid, which is believed to represent the rate-limiting step in TAG synthesis (Kinsella and Gross, 1973). Together, the data are suggestive of induction of de novo fatty acid synthesis and esterification by 16:0 through greater provision of substrate for the key enzymes *ACACA* and *FASN*, but also through greater acylation capacity. The latter effect might have involved binding and activation of PPARG.

In addition to the putative action of 16:0 through PPARG, increased TAG synthesis in mammary cells also could encompass upregulation in expression of the mammary-enriched LCFA-binding proteins *FABP3* and *FABP4*. In nonruminants, these *FABP* play a pivotal role in the trafficking and provision of LCFA for enzymes of TAG synthesis as well as the LCFA activation of PPARG (Mashek and Coleman, 2006). Such an effect in bovine mammary tissue was proposed previously for *FABP3* (Bionaz and Loor, 2008b). Upregulation

of *LPIN1*, a coactivator of PPARG in mouse adipose (Reue and Zhang, 2008), provides additional evidence that 16:0 might increase activation of PPARG. In this regard, the fact that PPARG apparently is able to bind 2 substrates simultaneously (Itoh et al., 2008) is suggestive of a need for an LCFA for full activation of the transcriptional program via ROSI. This might explain the lower than expected response to ROSI.

Because 16:0 is the main product of de novo synthesis in mammary cells, we propose that this LCFA could serve as a feed-forward mechanism for copious milk fat synthesis during the normal course of lactation. The large accumulation of lipid droplets (Figure 2 and Supplemental Figures 1 and 2; <http://jds.fass.org/content/vol92/issue9/>) supports this hypothesis. Overall, data indicate that 16:0 elicits a positive effect on milk TAG synthesis, partly through PPARG and potentially through as yet unidentified transcription factors (Figure 4). Duodenal infusion of palmitic acid resulted in greater milk fat synthesis and milk 4:0 concentration and yield (Enjalbert et al., 2000).

Stearic Acid. Although there was some overlap in responses between 18:0 and ROSI, current and previous data are suggestive of 18:0 as a less potent or “selective” ligand of PPARG. Stearate increased *INSIG1* and *AGPAT6* as well as *DGAT1*. Contrary to 16:0, however, 18:0 decreased de novo synthesis but increased mammary TAG synthesis from preformed fatty acids in mammary epithelial cell cultures (Hansen and Knudsen, 1987). We observed lower *ACACA* and *FASN* expression with 18:0 and other LCFA considered to be strictly of exogenous origin (e.g., *trans*-10 18:1, *t10c12* CLA, 20:5; Table 1 and Supplemental Figure 4). Biologically, a positive effect of 18:0 on mammary cell TAG synthesis (Hansen and Knudsen, 1987; Enjalbert et al., 2000) can be explained by the fact that this LCFA is primarily taken up from blood and not synthesized endogenously; that is, mammary cells reduce de novo synthesis in favor of esterification. In fact, greater *AGPAT6* mRNA with 18:0, but also with unsaturated LCFA (i.e., *cis*-9 18:1, *t10c12* CLA, and 20:5; Table 1), which are of exogenous origin, could be explained by the need to load additional LCFA into a growing intracellular pool of lysophosphatidic acid.

A previous study with MacT cells showed that exogenous 18:0 (25, 50, and 100 μ M) could have a positive effect on *ACACA* and *FASN* activity; that is, despite lower mRNA abundance, the efficiency of these reactions might somehow be enhanced by 18:0 (Jayan and Herbein, 2000). It also is possible that endogenous synthesis of *cis*-9 18:1 from absorbed 18:0 through *SCD* plays a role in driving greater TAG synthesis, as speculated previously (Loor and Herbein, 2003; Bionaz and Loor, 2008b). This idea is partly supported by the

significant formation of lipid droplets in cells cultured with 18:0 relative to the control (Figure 2 and Supplemental Figures 1 and 2), and also by the lack of change in *SCD* mRNA expression (Table 1) or *SCD* activity with incremental 18:0 (Jayan and Herbein, 2000). Exogenous 18:0 led to greater intracellular *cis*-9 18:1 in MacT cells (Jayan, 1998). The *SCD* was the most highly expressed transcript among those measured in MacT cells (Supplemental Figure 3). Overall, data suggest that 18:0 could manipulate de novo milk fatty acid synthesis through effects on gene expression.

Overall LCFA Regulation of Mammary Lipogenic Genes

Trans-10 Biohydrogenation Intermediates.

Effects of *t10c12* CLA in our experiment were similar to those in previous reports (Harvatine and Bauman, 2006; Bauman et al., 2008). However, genes such as *GPAM*, *LPL*, *AGPAT6*, and *ACSL1* were unaffected by this CLA. This can be explained by several factors, including cell culture conditions, measurement of different isoforms across studies (e.g., *AGPAT6* and *ACSL1*), use of a different approach for normalization of qPCR data, or their combination. Novel information in this regard is the negative effect of *t10c12* CLA on expression of additional lipogenic genes such as *FABP3* and *ACSS2*. Overall, our data confirmed that *t10c12* CLA has potent negative effects on lipogenic gene networks in mammary cells (Table 1 and Supplemental Figure 6). However, in our study, *t10c12* CLA induced greater intracellular TAG accumulation (Figure 2 and Supplemental Figures 1 and 2). We can infer a mechanism in which there was a high rate of uptake of CLA, coupled with its preferential distribution (and its metabolites) into the neutral lipid fraction (Banni et al., 2001). The former is supported by the large upregulation of *CD36* (which was common for all LCFA), as observed previously in similar in vitro studies (Yonezawa et al., 2008). Data on cell counts (data not shown) suggest that there was no apparent increase in apoptosis with *t10c12* CLA, in contrast with a previous report at a lower level of supplementation (Keating et al., 2008). A biological explanation for the large accumulation of lipid droplets with *t10c12* CLA despite downregulation of classical lipogenic genes is not apparent.

Although with different potency relative to *t10c12* CLA, *trans*-10 18:1 decreased expression of *FASN* and *SCD*, both of which are crucial for de novo fatty acid synthesis and generation of *cis*-9 18:1 for TAG formation (Loor and Herbein, 2003). Recently, a lack of effect of short-term (4-d) intestinally infused (approximately 43 g/d) *trans*-10 18:1 on milk fat synthesis in lactating cows was reported (Lock et al., 2007). However, milk

trans-10 18:1 reached only 1.1% of total fatty acids during infusions, well below the concentration calculated for maximal milk fat depression of 2.5% (Loor et al., 2005c; Kadegowda et al., 2008). Thus, higher amounts of *trans*-10 18:1 reaching the mammary gland could potentially induce milk fat depression by decreasing lipogenic gene expression. *Trans*-10 18:1 did not elicit effects similar to ROSI; thus, it does not appear to be a ligand of PPARG.

Oleic Acid. Oleic acid resulted in similar effects on expression of lipogenic genes as *t10c12* CLA (Table 1 and Supplemental Figure 4). This LCFA was the most potent inhibitor of *SCD* expression, which is suggestive of a feedback-inhibition mechanism. A previous study with MacT cells reported lower *ACACA*, *FASN*, and *SCD* activity with incremental exogenous *cis*-9 18:1 (25, 50, and 100 μ M; Jayan and Herbein, 2000). Exogenous oleic acid also inhibited acetate incorporation into TAG by dispersed ruminant mammary cells (Hansen and Knudsen, 1987). Our in vitro data suggest that *cis*-9 18:1 can potentially have a negative effect on lipoprotein utilization by mammary tissue (i.e., *LPL*), trafficking (i.e., *FABP3*), and desaturation (i.e., *SCD*). Compared with 16:0 and 18:0, oleic acid affected expression of only *AGPAT6* in a similar fashion as ROSI; thus, we can infer that this LCFA is a weak effector of PPARG.

Eicosapentaenoic Acid. Eicosapentaenoic acid reduced expression of genes involved in the nonruminant *SREBF1* network (Horton et al., 2003; Table 1 and Supplemental Figure 5). In addition to inducing lower mRNA of *SREBF1* and *SCAP*, expression of *INSIG1* increased. This latter effect might further decrease the activity of *SREBF1* (Kast-Woelbern et al., 2004). The decrease in *SCD* mRNA by 20:5 was lower relative to *t10c12* CLA. In this regard, it is noteworthy that activation of PPAR δ (via GW0742) increased *INSIG1* (mRNA and protein), inhibited proteolytic-processing of mature *SREBF1*, and reduced *FASN*, *ACACA*, and *SCD*, all of which led to reduced intracellular lipid accumulation in murine liver (Qin et al., 2008). Bovine mammary tissue expresses PPAR δ (M. Bionaz, S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, and J. J. Loor, University of Illinois, Urbana; unpublished results). Together, these data suggest that 20:5 could regulate mammary lipid synthesis through both PPARG and PPAR δ . Duodenal infusion of fish oil with high 20:5 content resulted in lower milk fat concentration as well as lower yield of 16:0 and *cis*-9 18:1 in milk fat (Loor et al., 2005b), all of which agree with the observed inhibitory effect of 20:5 on *SREBF1*, *SREBF2*, and *SCD*. Among unsaturated LCFA, 20:5 increased expression of *AGPAT6*, a PPARG target gene.

Relevance of Gene Expression Data to In Vivo Studies. Negative effects on milk fat synthesis in vivo have been convincingly demonstrated for *t10c12* CLA (Baumgard et al., 2000; Loor and Herbein, 2003) and mixtures of *trans*-18:1 LCFA (Gaynor et al., 1994). Despite the lack of “strong” in vivo data (see Chilliard et al., 2001), the fact that all unsaturated LCFA decreased (significantly or numerically) mRNA expression of *SREBF1*, *SREBF2*, *THRSP*, *SCD*, *ACACA*, and *LPL* indicates that they have some ability to reduce in vivo mammary synthesis of fatty acids, TAG, or both (Enjalbert et al., 2000; Loor et al., 2005b; Kadegowda et al., 2008). One likely mechanism stems from an “indirect” inhibition of *SREBF1* and *SREBF2*, that is, down-regulation through an upstream transcription factor or nuclear receptor able to bind unsaturated LCFA (Qin et al., 2008). This suggestion is supported by significant downregulation (and tendencies) of the *SREBF* target genes *ACACA*, *FABP3*, *LPIN1*, and *SCD* (Table 1). Inhibition of *SCD*, as assessed by lower *cis*-9 18:1/18:0 milk fatty acid ratios or *SCD* mRNA (Baumgard et al., 2002), is a classical marker of milk fat depression induced by exogenous *t10c12* CLA (Baumgard et al., 2000; Loor and Herbein, 2003).

CONCLUSIONS

Overall, results suggest that 16:0 could serve as a feed-forward mechanism to regulate the lipogenic program in bovine mammary cells, partly through PPARG activation. We recognize that mRNA expression data are only suggestive of an effect on PPARG activity by LCFA. Thus, more controlled molecular experiments, such as coin incubations of LCFA and a PPARG inhibitor or use of small interfering RNA to depress PPARG protein expression, will have to be performed in the future to address the role of LCFA action on PPARG fully. Future studies also need to investigate the balance of LCFA that is required for regulation of lipogenic gene networks via PPARG. In general, LCFA appear more likely to alter lipogenic gene expression through indirect effects (e.g., binding to other PPAR or nuclear receptors) on *SREBF1*- and *SREBF2*-mediated mechanisms. Availability of preformed LCFA, rather than de novo-synthesized fatty acids, was more important for lipid droplet formation, as indicated by the lack of accumulation of lipid droplets with ROSI despite the large availability of acetate and upregulation of *ACACA* and *FASN*. At the concentration used in this study, we observed that the negative effect on lipogenic gene expression with *trans*-10 18:1 was similar to that of *t10c12* CLA. Larger accumulation of TAG with *t10c12* CLA despite downregulation of lipogenic

genes merits further investigation. Ongoing functional genomics studies at the University of Illinois (Bionaz et al., 2007) highlight the complexity of the mammary transcriptome, posing a question regarding the involvement of additional transcriptional regulators (Figure 4 and Supplemental Figures 4 to 6; <http://jds.fass.org/content/vol92/issue9/>) in mammary lipid synthesis.

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REFERENCES

- Banni, S., G. Carta, E. Angioni, E. Murru, P. Scanu, M. P. Melis, D. E. Bauman, S. M. Fischer, and C. Ip. 2001. Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver. *J. Lipid Res.* 42:1056–1061.
- Bauman, D. E., J. W. Perfield 2nd, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: Lactation and the ruminant model. *J. Nutr.* 138:403–409.
- Baumgard, L. H., B. A. Corl, D. A. Dwyer, A. Saebø, and D. E. Bauman. 2000. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278:R179–R184.
- Baumgard, L. H., E. Matitashvili, B. A. Corl, D. A. Dwyer, and D. E. Bauman. 2002. *Trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. *J. Dairy Sci.* 85:2155–2163.
- Bennett, M. K., Y. K. Seo, S. Datta, D. J. Shin, and T. F. Osborne. 2008. Selective binding of sterol regulatory element-binding protein isoforms and co-regulatory proteins to promoters for lipid metabolic genes in liver. *J. Biol. Chem.* 283:15628–15637.
- Bensinger, S. J., and P. Tontonoz. 2008. Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* 454:470–477.
- Berger, J., and D. E. Moller. 2002. The mechanisms of action of PPARs. *Annu. Rev. Med.* 53:409–435.
- Bionaz, M., and J. J. Loo. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319.
- Bionaz, M., and J. J. Loo. 2008a. *ACSL1*, *AGPAT6*, *FABP3*, *LPIN1*, and *SLC27A6* are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. *J. Nutr.* 138:1019–1024.
- Bionaz, M., and J. J. Loo. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* 9:366.
- Bionaz, M., S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, and J. J. Loo. 2007. MammOmicsTM: Transcript profiling of the mammary gland during the lactation cycle in Holstein cows. *J. Dairy Sci.* 90:207–208.
- Carson, F. 1990. Histotechnology: A self-instructional text. ASCP Press, Chicago, IL.
- Chilliard, Y., A. Ferlay, and M. Doreau. 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest. Prod. Sci.* 70:31–48.
- Desvergne, B., L. Michalik, and W. Wahli. 2006. Transcriptional regulation of metabolism. *Physiol. Rev.* 86:465–514.
- Enjalbert, F., M. C. Nicot, C. Bayourthe, and R. Moncoulon. 2000. Effects of duodenal infusions of palmitic, stearic, or oleic acids on milk composition and physical properties of butter. *J. Dairy Sci.* 83:1428–1433.
- Fujino, T., J. Kondo, M. Ishikawa, K. Morikawa, and T. T. Yamamoto. 2001. Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. *J. Biol. Chem.* 276:11420–11426.
- Gaynor, P. J., R. A. Erdman, B. B. Teter, J. Sampugna, A. V. Capuco, D. R. Waldo, and M. Hamosh. 1994. Milk fat yield and composition during abomasal infusion of *cis* or *trans* octadecenoates in Holstein cows. *J. Dairy Sci.* 77:157–165.
- Genome Browser Gateway. 2008. <http://genome.ucsc.edu> Accessed June 23, 2008.
- Hansen, H. O., and J. Knudsen. 1987. Effect of exogenous long-chain fatty acids on individual fatty acid synthesis by dispersed ruminant mammary gland cells. *J. Dairy Sci.* 70:1350–1354.
- Harvatine, K. J., and D. E. Bauman. 2006. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. *J. Nutr.* 136:2468–2474.
- Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA* 100:12027–12032.
- Itoh, T., L. Fairall, K. Amin, Y. Inaba, A. Szanto, B. L. Balint, L. Nagy, K. Yamamoto, and J. W. Schwabe. 2008. Structural basis for the activation of PPARgamma by oxidized fatty acids. *Nat. Struct. Mol. Biol.* 15:924–931.
- Jayan, G. C. 1998. Activity and mRNA abundance of enzymes for fatty acid synthesis and desaturation in mammary cell cultures. PhD Diss. Virginia Tech, Blacksburg.
- Jayan, G. C., and J. H. Herbein. 2000. "Healthier" dairy fat using *trans*-vaccenic acid. *Nutr. Food Sci.* 30:304–309.
- Kadegowda, A. K., L. S. Piperova, and R. A. Erdman. 2008. Principal component and multivariate analysis of milk long-chain fatty acid composition during diet-induced milk fat depression. *J. Dairy Sci.* 91:749–759.
- Kast-Woelbern, H. R., S. L. Dana, R. M. Cesario, L. Sun, L. Y. de Grandpre, M. E. Brooks, D. L. Osburn, A. Reifel-Miller, K. Klausner, and M. D. Leibowitz. 2004. Rosiglitazone induction of Insig-1 in white adipose tissue reveals a novel interplay of peroxisome proliferator-activated receptor gamma and sterol regulatory element-binding protein in the regulation of adipogenesis. *J. Biol. Chem.* 279:23908–23915.
- Keating, A. F., F. Q. Zhao, K. A. Finucane, D. R. Glimm, and J. J. Kennelly. 2008. Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression. *Domest. Anim. Endocrinol.* 34:284–292.
- Kinsella, J. E., and M. Gross. 1973. Palmitic acid and initiation of mammary glyceride synthesis via phosphatidic acid. *Biochim. Biophys. Acta* 316:109–113.
- Kolak, M., H. Yki-Jarvinen, K. Kannisto, M. Tiikkainen, A. Hamsten, P. Eriksson, and R. M. Fisher. 2007. Effects of chronic rosiglitazone therapy on gene expression in human adipose tissue in vivo in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* 92:720–724.
- Lock, A. L., C. Tyburczy, D. A. Dwyer, K. J. Harvatine, F. Destailats, Z. Mouloungui, L. Candy, and D. E. Bauman. 2007. *Trans*-10 octadecenoic acid does not reduce milk fat synthesis in dairy cows. *J. Nutr.* 137:71–76.
- Loo, J. J., H. M. Dann, R. E. Everts, R. Oliveira, C. A. Green, N. A. Guretzky, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2005a. Temporal gene expression profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function. *Physiol. Genomics* 23:217–226.
- Loo, J. J., M. Doreau, J. M. Chardigny, A. Ollier, J. L. Sebedio, and Y. Chilliard. 2005b. Effects of ruminal or duodenal supply of fish oil on milk fat secretion and profiles of *trans*-fatty acids and conjugated linoleic acid isomers in dairy cows fed maize silage. *Anim. Feed Sci. Technol.* 119:227–246.

- Loor, J. J., A. Ferlay, A. Ollier, M. Doreau, and Y. Chilliard. 2005c. Relationship among *trans* and conjugated fatty acids and bovine milk fat yield due to dietary concentrate and linseed oil. *J. Dairy Sci.* 88:726–740.
- Loor, J. J., and J. H. Herbein. 2003. Reduced fatty acid synthesis and desaturation due to exogenous *trans*10,*cis*12-CLA in cows fed oleic or linoleic oil. *J. Dairy Sci.* 86:1354–1369.
- Mashek, D. G., and R. A. Coleman. 2006. Cellular fatty acid uptake: The contribution of metabolism. *Curr. Opin. Lipidol.* 17:274–278.
- Morandini, P., F. Salamini, and P. Gantet. 2005. Engineering of plant metabolism for drug and food. *Curr. Med. Chem. Immunol. Endocr. Metab. Agents* 5:103–112.
- National Center for Biotechnology Information. 2008. <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>.
- Pegorier, J. P., C. Le May, and J. Girard. 2004. Control of gene expression by fatty acids. *J. Nutr.* 134:2444S–2449S.
- Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2003. Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. *J. Nutr.* 133:3098–3102.
- Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2004. The inhibitory effect of *trans*-10, *cis*-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. *J. Nutr.* 134:2523–2527.
- Qin, X., X. Xie, Y. Fan, J. Tian, Y. Guan, X. Wang, Y. Zhu, and N. Wang. 2008. Peroxisome proliferator-activated receptor- δ induces insulin-induced gene-1 and suppresses hepatic lipogenesis in obese diabetic mice. *Hepatology* 48:432–441.
- Ramirez-Zacarias, J. L., F. Castro-Munozledo, and W. Kuri-Harcuch. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 97:493–497.
- Reue, K., and P. Zhang. 2008. The lipin protein family: Dual roles in lipid biosynthesis and gene expression. *FEBS Lett.* 582:90–96.
- Sauma, L., K. G. Stenkula, P. Kjolhede, P. Stralfors, M. Soderstrom, and F. H. Nystrom. 2006. PPAR- γ response element activity in intact primary human adipocytes: Effects of fatty acids. *Nutrition* 22:60–68.
- Sturn, A., J. Quackenbush, and Z. Trajanoski. 2002. Genesis: Cluster analysis of microarray data. *Bioinformatics* 18:207–208.
- Walkey, C. J., and B. M. Spiegelman. 2008. A functional peroxisome proliferator-activated receptor- γ ligand-binding domain is not required for adipogenesis. *J. Biol. Chem.* 283:24290–24294.
- Way, J. M., W. W. Harrington, K. K. Brown, W. K. Gottschalk, S. S. Sundseth, T. A. Mansfield, R. K. Ramachandran, T. M. Willson, and S. A. Kliewer. 2001. Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor γ activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269–1277.
- Wright, T. C., J. P. Cant, J. T. Brenna, and B. W. McBride. 2006. Acetyl CoA carboxylase shares control of fatty acid synthesis with fatty acid synthase in bovine mammary homogenate. *J. Dairy Sci.* 89:2552–2558.
- Yonezawa, T., M. Sanosaka, S. Haga, Y. Kobayashi, K. Katoh, and Y. Obara. 2008. Regulation of uncoupling protein 2 expression by long-chain fatty acids and hormones in bovine mammary epithelial cells. *Biochem. Biophys. Res. Commun.* 375:280–285.