Time-dependent effect of *trans*-10,*cis*-12 conjugated linoleic acid on gene expression of lipogenic enzymes and regulators in mammary tissue of dairy cows

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ABSTRACT

Trans-10,*cis*-12 conjugated linoleic acid (CLA) has been identified as an intermediate of rumen fatty acid biohydrogenation that caused milk fat depression (MFD) in the dairy cow. Previous studies in cows experiencing CLA- and diet-induced MFD have identified reduced mammary expression of the master lipogenic regulator sterol response element transcription factor 1 (*SREBF1*) and many of its dependent genes. To distinguish between primary mechanisms regulating milk fat synthesis and secondary adaptations to the reduction in milk fat, we conducted a time-course experiment. Eleven dairy cows received by abomasal infusion an initial priming dose of 6.25 g of CLA followed by 12.5 g/d delivered in multiple pulses per day for 5 d. Cows were milked 3×/d and mammary biopsies were obtained under basal condition (prebolus control) and 12, 30, and 120 h relative to initiation of CLA infusion. Milk fat concentration and yield decreased progressively reaching a nadir at 69 h (1.82% and 38.2 g/h) and averaged $2.03 \pm 0.19\%$ and 42.1 ± 4.10 g/h on the last day of treatment ($\pm$ standard deviation). Expression of fatty acid synthase (*FASN*) and lipoprotein lipase (*LPL*) were decreased at 30 and 120 h compared with control. Expression of *SREBF1* and *THRSP* were also decreased at 30 and 120 h compared with control. Additionally, we failed to observe changes in other factors, including peroxisome proliferator-activated receptor γ and liver x receptor β and milk fat globular membrane proteins, during CLA treatment. However, expression of milk fat globular membrane proteins were decreased after 14 d of diet-induced MFD in samples from a previous experiment, indicating a possible long-term response. The rapid decrease in lipogenic enzymes, *SREBF1*, and

THRSP provide strong support for their transcriptional regulation as a primary mechanism of milk fat depression.

Key words: milk fat, conjugated linoleic acid, *SREBF1*, *THRSP*, milk fat depression

INTRODUCTION

Milk fat synthesis is variable in dairy cows and responsive to nutritional factors. Specifically, diet-induced milk fat depression (MFD) represents a decrease in milk fat yield with no change in yield of milk or other milk components (see review by Bauman and Griinari, 2003). Milk fat depression is caused by unique intermediates from ruminal fatty acid (FA) biohydrogenation; *trans*-10,*cis*-12 CLA was the first of these unique biohydrogenation intermediates to be identified and is the most well studied (Baumgard et al., 2000).

During MFD, mammary lipogenic capacity is decreased and transcription of mammary genes involved in milk fat synthesis are coordinately downregulated (Baumgard et al., 2002). Decreased expression of sterol response element transcription factor 1 (*SREBF1*) and thyroid hormone responsive spot 14 (*THRSP*) have been reported during *trans*-10,*cis*-12 CLA and diet-induced MFD (Harvatine and Bauman, 2006a). The protein *SREBF1* is a master regulator of lipid synthesis and has been shown to be repressed by PUFA in liver of rodents and humans (Jump et al., 2005). Although its exact biochemical function is not known, spot 14 (*THRPS*) is nuclear protein that is closely associated with the regulation of FA synthesis in lipogenic tissues, including the mammary gland during MFD, and may function as a transcriptional co-activator (Cunningham et al., 1998; Chou et al., 2007, 2008). Additionally, studies performed in mammary epithelial cells have suggested roles for liver x receptor β (*LXRB*; Oppi-Williams et al., 2013; Harvatine et al., 2014) and peroxisome proliferator-activated receptor γ (*PPAR* γ ; Shi et al., 2014) in regulation of milk fat synthesis (reviewed by Bionaz et al., 2015).

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Virtually all previous gene expression studies have been performed in the context of fully established MFD and, accordingly, cannot distinguish causative mechanisms from secondary physiological adaptations to reduced milk fat synthesis (i.e., responses causing reduced lipogenesis vs. those only associated with reduced mammary lipogenesis). Our objective was to investigate the temporal expression of lipogenic enzymes, *SREBF1*, and *THRSP* in mammary tissue of lactating cows during CLA-induced MFD and our hypothesis was that *SREBF1* and *THRSP* are primary responses during induction of MFD.

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Samples were collected from 11 ruminally fistulated lactating dairy cows from the Cornell University Teaching and Research Center in 2 blocks. In the first block, 8 midlactation cows were assigned randomly to sampling sequence in a 2-period design. Mammary biopsies before the CLA bolus (CON) and 12 h after the abomasal CLA bolus infusion were collected during 1 period and mammary biopsies were collected 30 and 120 h after the bolus in the other period. Biopsies within period were taken from alternate rear quarters and periods were separated by 14 d to allow recovery. In the second block, 3 midlactation cows had biopsies collected sequentially from alternate rear quarters in a single period, with biopsies of the same quarter being at least 4 d apart.

Trans-10,*cis*-12 CLA was infused into the abomasum using a 0.5-cm i.d. line placed through the rumen cannula. An initial priming dose of 6.25 g of CLA was given at time zero, followed by 2.1 g every 4 h for 36 h and then 3.1 g every 6 h until 120 h. This provided 12.5 g/d of *trans*-10,*cis*-12 CLA, which is slightly over the maximally effective dose (~10 g/d; de Veth et al., 2004). The *trans*-10,*cis*-12 CLA methyl ester stock (BASF Corporation, Ludwigshafen, Germany) contained 88.3% total CLA (98% *trans*-10,*cis*-12 isomer), 6.8% palmitic acid, 2.7% oleic acid, and 2.0% stearic acid. Previous investigations have shown that the methyl ester and free FA forms of *trans*-10,*cis*-12 CLA are equally effective in inhibiting milk fat synthesis (de Veth et al., 2004).

Cows received a TMR fed once per day in the first block and twice per day in the second block. Feed ingredients were dried (55°C forced-air oven for 72 h), ground (Wiley mill with 1-mm screen; Arthur H.

Thomas, Swedesboro, NJ), and nutrient composition determined by wet chemistry procedures (AOCS, 2000; at Dairy One Cooperative, Ithaca, NY). The diet contained 31.1 and 33.2% NDF and 16.6 and 16.7% CP in the first and second block, respectively (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13935>).

Samples from a previous experiment (Harvatine and Bauman, 2006a) were used to further investigate the regulation of the milk fat globular membrane (MFGM) proteins during MFD. Briefly, mammary biopsies were collected as described above from control (normal milk fat) and after 3 d of intravenous infusion of 10 g/d of *trans*-10,*cis*-12 CLA that resulted in a 24% decrease in milk fat yield, and 10 d of a low-forage and high-oil diet (3.0% soybean oil and 1.5% fish oil) that resulted in a 38% reduction in milk fat yield.

Sampling and Analysis

Cows were milked 3×/d (0800, 1600, and 2400 h) and samples were taken at times shown and analyzed for fat and true protein using a mid-infrared spectrophotometer (AOCS, 2000; at Dairy One Cooperative). Additionally, milk lipids were extracted in hexane-isopropanol, transmethylated with sodium methoxide, and FAME were quantified by GC according to Perfield et al. (2006). Transfer of CLA to milk was calculated as yield in milk divided by amount infused.

Mammary biopsies were performed at approximately 1030 h according to (Harvatine and Bauman, 2006a) using a needle biopsy tool (Magnum Biopsy Gun system; Bard Biopsy Systems, Tempe, AZ). Tissues were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from approximately 30 mg of mammary tissue using the RNeasy Lipid Kit with on column Dnase I (Qiagen, Valencia, CA). The RNA concentration and integrity were determined by an Agilent 2100 BioAnalyzer [RNA integrity number 8.4 ± 0.46 (mean $\pm$ SD); Agilent Technologies, Santa Clara, CA]. Total RNA was reverse transcribed using the SuperScript III First Strand Synthesis kit (Invitrogen, Carlsbad, CA) with random primers (3 μ g of total RNA in a 20 μ L total reaction with 2.5 ng/ μ L of primers). Quantitative real-time reverse transcriptase PCR (qRT-PCR) assays were developed and validated for genes of interest (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13935>), as described by Harvatine and Bauman (2006a). The qRT-PCR reactions included ABI Power SYBR with ROX (Applied Biosystems, Foster City, CA), 400 nM gene-specific forward and reverse primers (Invitrogen), and 5 to 25 ng of cDNA in a 2-step amplification program (95°C for 15 s and 60°C for 60 s) with an ABI PRISM 7000 Sequence Detection System

(Applied Biosystems). Dissociation curves were generated at the end of amplification to verify presence of a single product. Sample message level was determined relative to a dilution curve of pooled mammary cDNA (ABI Prism, 2001).

The mammary samples from (Harvatine and Bauman, 2006a) were handled similarly as described above and qRT-PCR was used to determined expression of *CD36* molecule (*CD36*), butyrophilin subfamily 1 member A1 (*BTN1*), perilipin 2 (*PLIN2*; previously called adipophilin), xanthine dehydrogenase (*XDH*; commonly called xanthine oxidoreductase), and mucin 1 (*MUC1*).

Statistical Analysis

Data from the time course experiment were analyzed using the fit model procedure of JMP Pro (SAS Institute Inc., Cary, NC). The model to test treatment means included the random effect of cow and period and the fixed effect of time after the CLA priming dose. Additionally, the geometric mean of 3 reference genes [ribosomal protein S9 (*RPS9*), β -actin (*ACTB*), and β 2-microglobulin (*B2M*)] was calculated using GeNorm (Vandesompele et al., 2002) and used as a covariant in the model (Allison et al., 1995; Martinez et al., 2004). Data were log-transformed when necessary, and back-transformed data are reported. Means and standard errors were scaled with control scaled to equal 100 after statistical analysis. Preplanned contrasts compared gene expression of CON versus 12 h, CON versus 30 h, and CON versus 120 h. Expression of the MFGM proteins in the samples from Harvatine and Bauman (2006a) were similarly modeled with the effect of treatment rather than time after bolus, and preplanned contrast was control versus CLA and control versus low-forage and high-oil diet.

RESULTS AND DISCUSSION

Milk *trans*-10,*cis*-12 CLA and Milk Fat Synthesis

Milk fat concentration of *trans*-10,*cis*-12 CLA was 0.25% of total FA at 13 h after the bolus and averaged $0.28 \pm 0.021\%$ ($\pm$ SD) from 29 to 117 h after the bolus (Figure 1A). Transfer efficiency over the last day of treatment was 22.9% (2.8 g/d transferred), which is similar to the 21.8% transfer efficiency (2.7 g/d transferred) predicted by the meta-regression of de Veth et al. (2004).

Milk fat concentration and yield decreased progressively and was lower than control by 21 h after the bolus (Figure 1C and D). A nadir was observed in milk fat concentration and yield at 69 h (1.82% and 38.2 g/h, respectively) and averaged $2.03 \pm 0.19\%$ and $42.1 \pm$

4.10 g/h on the last day of treatment, respectively. This represented a 40% reduction in milk fat concentration and a 46% reduction in milk fat yield. This reduction is very similar to previous work, as the summary by de Veth et al. (2004) predicts the 12.5-g/d dose would decrease milk fat yield by 47%.

The response to CLA occurred in 2 phases and varied according to the origin of the FA (Figure 1B). The concentration of preformed FA (>16 C) was 38.9% of FA before the time-zero bolus and first increased to $40.9 \pm 0.35\%$ FA between 13 and 29 h and then further increased to $46.3 \pm 0.46\%$ of FA after 69 h on treatment. De novo-synthesized FA tended to be decreased at 21 h and were decreased 5.4 percentage points after 69 h compared with before the bolus. Fatty acids of mixed origin (16 C) were 30.9% before the time-zero bolus and initially decreased to $29.5 \pm 0.03\%$ of FA from 13 to 29 h and then decreased to $28.6 \pm 0.64\%$ of FA after 69 h. We had previously observed a similar 2-phase response with the same CLA infusion protocol during every 4-h milking and proposed that the first phase is a decrease in both de novo and preformed FA, whereas the second phase is a greater reduction in de novo synthesis (Harvatine and Bauman, 2011). The timing of the biopsies (12, 30, and 120 h) was selected to provide tissue during critical points during these phases. Yield of all categories of FA were decreased by 13 h after initiation of CLA infusion (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13935>).

Milk Fat Synthesis and Expression of Lipogenic Enzymes

Mammary expression of *FASN* and *LPL* were decreased over 30% at 30 h after the bolus, but expression of *SCD1* was not changed (Figure 2A). These genes are transcriptionally regulated and were selected to characterize the effect on de novo synthesis, preformed FA pathways, and FA desaturation, respectively (Sul and Wang, 1998; Mauvoisin and Mounier, 2011; Kersten, 2014). A coordinated downregulation of lipogenic enzymes, including *FASN* and *LPL*, was consistently observed in mammary tissue when CLA and diet-induced MFD plateaued (Harvatine and Bauman, 2006a). Vyas et al. (2013) also reported decreased protein abundance of *FASN* and acetyl-CoA synthase in mammary tissue during CLA treatment. Although the delta-9 desaturase ratio (product-precursor relationship) consistently decreased during CLA-induced MFD, *SCD1* expression is variable; Harvatine and Bauman (2006a) reported no change, whereas Gervais et al. (2009) reported a tendency to decrease and Han et al. (2012) reported a decrease in *SCD1* expression during CLA-induced MFD. The rapid decrease in lipogenic enzymes provides

additional support for a strong role of transcriptional regulation during MFD.

Expression of *SREBF1* and *THRSP*

Mammary expression of *SREBF1* was decreased 31% at 30 h and 48% at 120 h after the time-zero bolus (Figure 2B). Protein SREBF1 is a master regulator of lipid synthesis, and one key point of its regulation is protein cleavage resulting in the transcriptionally active nuclear fragment (Jump et al., 2005). However, active SREBF1 positively regulates its own regulation, making *SREBF1* expression indicative of SREBF1 activity (Jump et al., 2005). Decreased abundance of the active nuclear fragment was originally reported by Peterson et al. (2004) in mammary cell culture, and transcriptional downregulation of *SREBF1* in vivo was reported by Harvatine and Bauman (2006a) and subsequently verified by others (e.g., Gervais et al., 2009; Han et

al., 2012; Vyas et al., 2013). Li et al. (2014) reported that overexpression of *SREBF1* increased milk fat and inhibition decreased milk fat in bovine mammary epithelial cell culture and Ma et al. (2014) reported that *trans*-10,*cis*-12 CLA inhibited a reporter construct that contained the SREBP response element.

Mammary expression of *THRSP* was also decreased over 41% at 30 and 120 h after the bolus compared with control (Figure 2B). Originally *THRSP* was identified as a CLA-responsive gene in bovine mammary epithelial cell culture (Peterson et al., 2004) and was later reported to be repressed in the mammary gland during diet and CLA-induced MFD (Harvatine and Bauman, 2006a). Overexpression of *THRSP* was reported to increase fat synthesis in bovine mammary epithelial cells (Cui et al., 2015).

The protein PPARGC1 α is a transcriptional co-activator that was not changed by CLA, but was decreased by 14 d of diet-induced MFD (Harvatine and Bauman,

Figure 1. Temporal response of milk fat and milk fatty acid (FA) profile during abomasal infusion of 12.5 g/d of *trans*-10,*cis*-12 CLA for 5 d. (A) Concentration of *trans*-10,*cis*-12 CLA in milk fat; (B) concentration of FA less than 16 carbons (<16 C), 16 C, and >16 C in milk fat; (C) milk fat concentration; and (D) milk fat yield. Values are LSM $\pm$ SEM. *Asterisks indicate difference from prebolus level ($P < 0.05$). ‡ indicates a tendency ($P < 0.10$).

Figure 2. Temporal expression of genes in the mammary gland during abomasal infusion of 12.5 g/d of *trans*-10,*cis*-12 CLA for 5 d [before infusion, control (CON), and 12, 30, and 120 h after infusion]. (A) Selected lipogenic enzymes including fatty acid synthase (*FASN*), lipoprotein lipase (*LPL*), and stearoyl CoA desaturase (*SCD1*); (B) expression of sterol response element binding protein 1 (*SREBF1*), thyroid hormone responsive spot 14 (*THRSP*), and peroxisome proliferative activated receptor gamma coactivator 1 α (*PPARGC1α*); (C) expression of peroxisome proliferator-activated receptor γ (*PPARγ*), liver x receptor β (*LXRB*), and ATP binding cassette subfamily A member 1 (*ABCA1*). Values are LSM $\pm$ SEM ($n = 11$). Asterisks indicate difference from control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2006b). Mammary expression of *PPARGC1A* was not changed by CLA treatment in the current experiment, indicating it may either be responsive to other bioactive FA produced during diet-induced MFD or to longer durations of MFD.

Functional roles for both *SREBF1* and *THRSP* have been reported in transgenic mouse models and bovine mammary cell culture. Mammary disruption of *SCAP*, which is required for *SREBF1* activation, decreases pup growth and milk fat concentration (Rudolph et al., 2010), and whole body disruption of *THRSP* decreases milk fat concentration approximately 50% (Zhu et al., 2005). Demonstrating functional roles of genes in vivo is difficult in the cow, as transgenic models are not feasible and cell culture models do not recapitulate lactation because they fail to export synthesized lipids (Collier et al., 1977); however, improved 3-dimensional culture models may resolve some of these issues (Campbell and Watson, 2009). The decreased expression of *SREBF1* at 30 h in the current experiment indicates it is an early phase response.

A key question that remains is the mechanism linking CLA and downregulation of *SREBF1* and *THRSP* as neither protein has ligand binding domains. Lee et al. (2010) identified Ubx8 as an ER associated sensor protein that changed conformation in the presence of UFA and resulted in increased proteolysis of INSIG1, but the interaction of CLA with UBXD8 is not known. *SREBF1* regulation is thought to be dependent on membrane composition, especially in response to sterol concentration (Hagen et al., 2010).

Expression of *PPARγ* and *LXRB* and Target Genes

Members of the nuclear hormone receptor family could be regulators of lipid synthesis in the mammary gland and regulated by CLA, as they have ligand-binding pockets and are predominantly regulated by ligand activation. Although liver x receptor (*LXR*) agonists increase lipid synthesis in mammary epithelial cells, Harvatine et al. (2014) reported that neither *LXR* isoform or their specific target genes were modified during CLA or diet-induced MFD. No effect of CLA on *LXRB* or ATP-binding cassette subfamily A member 1 (*ABCA1*), a target gene highly regulated by *LXR* activation, was observed in the current experiment. An early investigation of CLA in other models proposed a role of modification of *PPARγ* signaling (Yu et al., 2002). A role of *PPARγ* in mammary regulation of lipid synthesis has been proposed (Bionaz and Loores, 2008). In the current experiment, *PPARγ* was not modified by CLA. Interestingly, in rodent models, *PPARγ* was activated by *trans*-10,*cis*-12 CLA in a transactivation assay, but *cis*-9,*trans*-11 CLA, which does not cause

milk fat depression, also activated PPAR γ (Moya-Camarena et al., 1999; Yu et al., 2002). Ma et al. (2014) also reported that *trans*-10,*cis*-12 CLA did not inhibit

activation of promoters with PPAR and LXR response elements in mammary epithelial cells.

Expression of Milk Fat Globular Membrane Proteins

Milk fat is assembled into lipid droplets and surrounded by phospholipids and specific proteins that form the MFGM and are essential for the fat secretion from the mammary epithelial cell (Bauman et al., 2006; Cavaletto et al., 2008). Expression of the MFGM genes has been generally overlooked in investigation of MFD. As they are essential, it is possible that a specific down-regulation would result in a limitation of milk fat secretion and an inhibition of fat synthesis. We determined the expression of *MUC1*, *PLIN2*, and *XDH* in the current study (Figure 3), as they are highly abundant in the MFGM and are thought to have essential roles in lipid secretion (Mather et al., 2001; Ogg et al., 2004; McManaman, 2012); however, their expression was not changed at any time point. To expand our investigation of the MFGM, we examined expression in mammary samples from Harvatine and Bauman (2006a), who investigated MFD induced by a 3-d i.v. treatment of CLA or feeding a low-forage and high-oil diet for 14 d. Similarly, we found no effect of CLA on the MFGM genes, including *CD36*, *BTN1A1*, *PLIN2*, *XDH*, and *MUC1*. However, diet-induced MFD decreased expression of *PLIN2* and *MUC1* and tended to decrease *CD36* and *XDH*. The reduction during diet-induced MFD may be related to other ruminally produced bioactive isomers or to the longer duration of MFD (14 d for diet-induced MFD vs. 3 and 5 d for CLA treatments). In either case, a reduction in MFGM is not required for induction of MFD and is expected to be a secondary long-term adaptation to reduced milk fat yield if it does occur.

CONCLUSIONS

The rapid decrease in lipogenic enzymes, *SREBF1*, and *THRSP* during CLA-induced MFD provides strong support for transcriptional regulation of these genes being a primary mechanism of MFD. Roles for the nuclear hormone receptors *PPARY* and *LXRB* and milk fat globular membrane proteins in CLA-induced MFD was not observed.

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Figure 3. Expression of milk fat globular membrane proteins during milk fat depression in 2 experiments. (A) Temporal expression of mucin 1 (*MUC1*), perilipin 2 (*PLIN2*, also known as adipophilin), and xanthine dehydrogenase (*XDH*, commonly called xanthine oxidoreductase) in mammary tissue during abomasal infusion of 12.5 g/d of *trans*-10,*cis*-12 CLA for 5 d [before infusion, control (CON), and 12, 30, and 120 h after infusion]. (B) Expression of *CD36* molecule (*CD36*), butyrophilin subfamily 1 member A1 (*BTN1A1*), *PLIN2*, *XDH*, and *MUC1* during control (CON), 3-d intravenous infusion of 10 g/d of *trans*-10,*cis*-12 CLA that resulted in a 24% decrease in milk fat, and 14 d of a low-forage and high-oil diet (LF/HO; 3.0% soybean oil and 1.5% fish oil) that resulted in a 24% decrease in milk fat. Values are LSM $\pm$ SEM. Differences compared with control are indicated ($\ddagger P < 0.10$, $*P < 0.05$).

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