Short communication: Effect of *trans*-10,*cis*-12 conjugated linoleic acid on activation of lipogenic transcription factors in bovine mammary epithelial cells

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ABSTRACT

The objective of this study was to examine the effect of *trans*-10,*cis*-12 conjugated linoleic acid (*t10c12CLA*) on the activation of transcription factors that potentially regulate lipid synthesis in a bovine mammary epithelial cell line (MAC-T). Cells were transfected with luciferase reporter constructs containing sterol response element (SRE and SRE complex) for sterol regulatory element binding protein-1, peroxisome proliferator response element for peroxisome proliferator-activated receptor γ , or liver X receptor response element for liver X receptor. Different concentrations of *t10c12CLA* (0, 25, 50, 75, or 100 μ M) were applied to cells to determine the activation of transcription factors. The influence of *t10c12CLA* bond structure on transcription factor activation was also investigated by treating cells with different 18:1 fatty acid isomers (*trans*-10 18:1 or *cis*-12 18:1) at 100 μ M. Cells were harvested for luciferase assay after 24 h of treatment. Compared with linoleic acid and *cis*-9,*trans*-11 CLA controls, the SRE reporters had significantly lower activity in *t10c12CLA*-treated cells at 50 and 75 μ M for SRE complex and SRE, respectively. Lower SRE and SRE complex activation was observed in *t10c12CLA* treatment at 25, 50, and 75 μ M compared with 0 μ M. The peroxisome proliferator response element and liver X receptor response element reporters did not respond differently between the *t10c12CLA* treatment and controls. Compared with *t10c12CLA*, both *trans*-10 18:1 and *cis*-12 18:1 increased the activities of SRE and SRE complex reporters by 1.3- to 4.2-fold. In conclusion, *t10c12CLA* has an inhibitory role in lipogenic transcription factor activation of SRE, and this negative effect is due to the conjugation of *trans*-10 and *cis*-12 double bonds in the fatty acid. Furthermore, we found no support for a regulatory role of response elements for peroxisome

proliferator-activated receptor γ or liver X receptor in the *t10c12CLA* inhibition of mammary lipid synthesis.

Key words: transcription factor, luciferase reporter, *trans*-10,*cis*-12 conjugated linoleic acid

Short Communication

Milk fat is an important component of milk and is decreased during milk fat depression (MFD). This reduction in milk fat is caused by FA intermediates of unsaturated FA biohydrogenation including *trans*-10,*cis*-12 CLA (*t10c12CLA*; Baumgard et al., 2000; Bauman and Grinari, 2001, 2003). Evidence shows decreased expression of fatty acid synthase and stearoyl-CoA desaturase-1 (SCD1) in the mammary gland of cows with abomasal *t10c12CLA* infusion (Baumgard et al., 2002). Additionally, the expression of sterol regulatory element (SRE) binding protein-1 (SREBP-1), a transcription factor (TF) involved in lipogenesis regulation, is reduced in *t10c12CLA*-treated bovine mammary epithelial cells and mammary tissue of infused cows, and promoter analysis has revealed binding sites for SREBP-1 in the bovine fatty acid synthase and SCD1 promoters (Peterson et al., 2004; Harvatine and Bauman, 2006). Therefore, *t10c12CLA* likely regulates lipogenic enzyme expression through SREBP-1, and knockdown of SREBP-1 reduces lipogenic gene expression in bovine mammary epithelial cells (Ma and Corl, 2012).

In the current study, peroxisome proliferator-activated receptor γ (PPAR γ) and liver X receptor (LXR) were 2 other TF of interest. Induction of lipogenic gene expression by a synthetic PPAR γ agonist has been observed in bovine mammary epithelial cells (Kadegowda et al., 2009), and an inhibitory effect of *t10c12CLA* on PPAR γ and one of its targets, lipoprotein lipase, has been described in mouse white adipose tissue (Kang et al., 2003). Together, these studies indicate some potential for PPAR γ as a TF regulating milk fat synthesis during MFD. Liver X receptor regulates SREBP-1 expression in mouse liver, adipose tissue, and intestine (Repa, 2000). It has also been shown that LXR ac-

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tivation upregulates the expression and activation of SREBP-1 in bovine mammary epithelial cells, suggesting potential regulation of milk fat synthesis by LXR (McFadden and Corl, 2010). Taken together, these TF are additional potential regulators and control points of FA synthetic pathways during MFD.

We hypothesized that *t10c12CLA* had an inhibitory effect on the activation of lipogenic TF in bovine mammary epithelial cells. In the current study, luciferase reporter constructs containing response elements for SREBP-1 (SRE and SRE complex), PPAR γ (**PPRE**), and LXR (**LXRE**) were transfected into bovine mammary epithelial cells to investigate the basic mechanisms of TF activation involved in the regulation of gene transcription by FA. Initially, cells were treated with *t10c12CLA* to evaluate its effect on TF activation. To further understand the regulation of TF activation by *t10c12CLA*, individual 18:1 FA with *t10* or *c12* double bond were used to treat transfected cells to investigate the effect of *t10c12CLA* conformation on TF activation. We chose to study the SRE complex reporter because the complex includes the binding sites of additional TF necessary for full transcriptional activation. Additionally, this complex is present in the promoter of SREBP-1 and might better reflect the self-regulation of SREBP-1 in a feed-forward mechanism. Understanding gene regulation by TF can be complicated by the presence of multiple TF elements in the gene promoter and interactions among them. The current study used tandem repeats of individual TF response elements to investigate the response of specific TF to FA treatments.

Oligonucleotides containing overhangs for NheI and XhoI and with tandem TF binding sites were commercially synthesized (Sigma, St. Louis, MO), including 4 copies of SRE (forward: 5'-ctagatcacccacatcacccacatcacccacatcacccac; reverse: 5'-tcgagtggggtgatgtggggtgatgtggggtgatgtggggtgat); 3 copies of LXRE (forward: 5'-ctagcgcgcgtagtaaccctgaccgccaagtaacccgcccgtagtaaccc; reverse: 5'-tcgagggttactagcgggcttactggcggtcagggttactagcgggctg); 3 copies of PPRE (forward: 5'-ctagaactgggccaaggtctaactgggccaaggtctaactgggccaaggtct; reverse: 5'-tcgaagaccttggccagtagaccttggccagtagaccttggccagtt); and an SRE complex containing NFY, Sp1, and SRE sites (forward: 5'-ctagtcattggcctggcgcgagccaagctgtcagccatgtggcgtgtcc; reverse: 5'-tcgaggacacgccatgggctgacagcttgctgcgcgccagggccaatga). The oligos were cloned into the pGL4.23[luc2/minP] firefly luciferase reporter vector containing a minimal promoter (Promega, Madison, WI) using the NheI and XhoI restriction sites.

Bovine mammary epithelial (**MAC-T**) cells were maintained in basal culture media consisting of Dulbecco's modified Eagle's medium—high glucose (Sigma)

with 10 kU/mL penicillin, 10 mg/mL streptomycin, and 25 μ g/mL amphotericin (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and routinely cultured at 37°C in 5% CO₂. For experiments, cells were seeded in 24-well plates at a density of 2×10^4 cells/cm². After overnight incubation in basal culture media containing 10% serum, cells were treated as described in experiments. Immediately after treatment application, cells were cotransfected with 0.5 μ g of the indicated pGL4.23[luc2/minP] reporter constructs and 30 ng of renilla luciferase control vector (hRLuc-TK, Promega) per well, using jetPEI transfection reagent (PolyPlus Transfection, New York, NY).

To examine the effects of CLA concentrations on TF reporter constructs, cells were treated with linoleic acid (**LNA**), *cis*-9,*trans*-11 CLA (**c9t11CLA**), or *t10c12CLA* (Nu-Chek Prep Inc., Elysian, MN) at 0 (BSA control), 25, 50, 75, or 100 μ M. Fatty acids were bound to BSA (Sigma) using the method described by Ip and coworkers (1999) with modifications. Briefly, a 30-mM FA-salt stock solution was prepared using pure FA and 0.05-M NaOH. Fatty acid salts were combined with a 100- μ M BSA solution to make a 300- μ M BSA-FA salt complex (3:1 FA-to-BSA molar ratio). To examine the role of double-bond structure on TF reporter constructs, cells transfected with reporters containing either SRE or SRE complex were treated with *t10c12CLA*, *t10* 18:1 (synthesized and purified as previously referenced; Lock et al., 2007), or *c12* 18:1 (Cayman Chemical, Ann Arbor, MI) at 100 μ M. *Cis*-9 (**c9**) and *trans*-11 (**t11**) 18:1 (Nu-Chek) were used as conformational controls. For BSA (0 μ M) control, no FA were added and BSA concentration was 17 μ M for the FA concentration experiment and 33 μ M for the FA double-bond experiment.

After 24 h of treatment, cells were washed with PBS, lysed, and assayed for firefly and renilla luciferase activity using the Dual Luciferase Reporter Assay (Promega) and a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA). Firefly luciferase values were normalized to renilla luciferase values for each sample. Each experiment was repeated 3 times.

The FA concentration data (0 μ M, BSA control used as a covariate) were fit to a linear model, and slopes (luciferase activity/FA concentration) were calculated by the general linear model procedure of SAS (SAS 9.2; SAS Institute Inc., Cary, NC). The contrast between LNA + *c9t11CLA* and *t10c12CLA* was constructed for slopes. Means at each concentration were compared with 0 μ M (BSA control) for each FA using the Dunnett's multiple comparison. Differences between treatment and control means at each concentration were determined using the Tukey's multiple comparison. Data examining the role

Figure 1. The effect of *trans*-10,*cis*-12 CLA (*t10c12CLA*) on luciferase reporter activities [A: sterol response element (SRE); B: SRE complex; C: peroxisome proliferator response element (PPRE); D: liver X receptor response element (LXRE)] driven by lipogenic transcription factor in bovine mammary epithelial (MAC-T) cells. Bovine mammary epithelial cells were treated with linoleic acid (LNA); *cis*-9,*trans*-11 CLA (*c9t11CLA*); or *t10c12CLA* at 0, 25, 50, 75, or 100 μ M, followed by cotransfection with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 μ g/well) containing SRE, SRE complex, PPRE, or LXRE in the reporter. After 24 h of transfection, cells were lysed and luciferase activities were measured. Each experiment was repeated 3 times. Luciferase activity (means $\pm$ SE) was calculated by the ratio of firefly luciferase activity to renilla luciferase activity. Difference was considered significant when $P < 0.05$.

of double-bond structure in the second experiment were analyzed using general linear model procedure. Means of each FA and the BSA control and means of each 18:1 FA and *t10c12CLA* treatment were compared in separate analyses using Dunnett's multiple comparison. Differences were considered significant when $P < 0.05$.

The first experiment investigated the regulation of TF activation by FA by comparing *t10c12CLA* with 2 control FA. Luciferase activity for reporters containing SRE and SRE complex was reduced by *t10c12CLA* by 22 to 34% (25–75 μ M, $P < 0.05$), but 100 μ M was not different compared with BSA control (Figure 1A and B). However, the reporter activities in *t10c12CLA*-treated cells were significantly lower than *c9t11CLA* (SRE) or both LNA and *c9t11CLA* (SRE complex) at 75 and 50 μ M, respectively ($P < 0.01$). Lower concentrations of LNA and *c9t11CLA* inhibited activation of the SRE reporter to some extent compared with the BSA control, but activation of both reporters increased with increasing concentrations of *c9t11CLA* or both

LNA and *c9t11CLA* and were significantly greater than *t10c12CLA* ($P < 0.01$, Figure 1A and B).

Previous studies have reported decreased SREBP-1 proteolytic activation and reduced expression of fatty acid synthase synthase and SCD1, 2 enzymes involved in lipid synthesis with SRE sequences in their promoters, by *t10c12CLA* at 75 μ M in MAC-T cells (Peterson et al., 2004). Similarly, low concentrations (15 and 30 μ M) of *c9t11CLA* and *t10c12CLA* have been shown to decrease SCD1 transcriptional activity in MAC-T cells, possibly through SREBP-1, which directly regulates SCD1 transcription (Keating et al., 2008; Ma and Corl, 2012). Our observations are consistent with these reports and support a regulatory role for SREBP-1 in *t10c12CLA*-induced inhibition of lipogenesis. The reduction of luciferase activity for SRE reporters could be due to decreased DNA binding activity of SREBP-1 or decreased expression of the TF. The mRNA expression of SREBP-1 and protein expression of the precursor form of SREBP-1 were unchanged by *t10c12CLA*; how-

ever, protein expression of the active nuclear form of SREBP-1 was decreased 26% by *t10c12CLA* in MAC-T cells (Peterson et al., 2004). In contrast, Kadegowda et al. (2009) and Harvatine et al. (2014) reported a decrease in SREBP-1 mRNA expression in MAC-T cells treated with *t10c12CLA*, but they did not report protein expression results for either the precursor or mature form of SREBP-1. In the current study, whether the lower luciferase activity of SRE reporters with *t10c12CLA* treatment is attributable to decreased protein expression of SREBP-1 or decreased activation of the precursor form to the transcriptionally active, mature form cannot be discerned. Many studies use *c9t11CLA* as a control when investigating the role of *t10c12CLA* in mammary lipogenesis and show an inhibitory effect of *t10c12CLA* on SREBP-1 and lipid synthesis (Baumgard et al., 2000; Peterson et al., 2004). Our results do not conflict with previous reports by showing lower SRE reporter activity with *t10c12CLA* treatment compared with *c9t11CLA*, although these FA either increased or did not change SREBP-1 activation at 100 μ M compared with BSA control as indicated by lack of change in luciferase activation. We also used LNA as a control in the current study because LNA and *t10c12CLA* are similar in having 2 double bonds and sharing a *cis-12* double bond. Moreover, using LNA as a control excluded the complicity of double-bond conjugation. A previous study has shown that LNA decreases SREBP-1c promoter activity in cell culture, which might explain the similar effect of LNA and *t10c12CLA* on SRE reporter activation (Yoshikawa et al., 2002). However, the different effects of LNA on SRE and SRE complex reporters were probably due to interactions between LNA and other elements in the SRE complex and requires examination for verification.

Luciferase activity of the PPARE reporter increased linearly by *t10c12CLA*, LNA, and *c9t11CLA* ($P < 0.05$) and was significantly greater at 100 μ M compared with BSA control, but no difference was found for the increasing rate or reporter activity between *t10c12CLA* treatment and LNA or *c9t11CLA* (Figure 1C). The LXRE reporter activity was inhibited by *t10c12CLA* at 25 μ M compared with BSA control and increased moderately at higher concentrations ($P < 0.05$). Similarly, its activity was reduced by LNA (25 μ M) and *c9t11CLA* (25 and 50 μ M) compared with BSA control and increased linearly at higher concentrations (Figure 1D); however, no difference between *t10c12CLA* and LNA or *c9t11CLA* was observed at any concentration.

Although the responses of PPAR γ and LXR to MFD have not been fully examined, studies have shown co-ordinated upregulation of lipogenic genes when the 2 TF are activated by their agonists in bovine mammary epithelial cells, indicating their potential involvement in

the regulation of milk fat synthesis (Kadegowda et al., 2009; McFadden and Corl, 2010). As nuclear receptors, PPAR γ and LXR can bind to FA and are regulated by FA (Pawar et al., 2002). Evidence shows an inhibitory effect of *t10c12CLA* on PPAR γ expression and activation in adipose tissue (Tsuboyama-Kasaoka et al., 2000; Kennedy et al., 2008); however, the PPARE reporter response to *t10c12CLA* in our study indicates little negative influence on its activation in cultured mammary epithelial cells. In fact, *t10c12CLA* has been found to be an activator and ligand for PPAR γ in kidney cells (Belury et al., 2002). Linoleic acid and *c9t11CLA* are also recognized as ligands and agonists of PPAR γ (Xu et al., 1999; Villacorta et al., 2009). In bovine mammary epithelial cells, the PPARE reporter activation by the 3 FA might be due to their agonistic role in PPAR γ activation, and this hypothesis needs to be examined by further studies. For the mRNA expression of PPAR γ , Kadegowda et al. (2009) reported no change in MAC-T cells treated with *t10c12CLA*. Liver X receptor also regulates the expression of SREBP-1 and binding sites exist for LXR in bovine SREBP-1c promoter (Lengi and Corl, 2010; McFadden and Corl, 2010). Previous studies led to our hypothesis that SREBP-1 reduction during MFD might be regulated by LXR. In the current study, LXRE reporter activation was inhibited by *t10c12CLA* at low concentration, but similar inhibition was also observed with the 2 FA controls, suggesting that the inhibition was likely a broad response of the vector to FA in MAC-T cells. A recent study shows that LXR knockdown does not change the expression of lipogenic enzymes in MAC-T cells, indicating that LXR reduction might not be required for lipogenic enzyme and lipid synthesis inhibition by *t10c12CLA* (Oppi-Williams et al., 2013). Although a recent study reported unchanged LXR mRNA expression in CLA-treated cows, the lack of response in MAC-T cells cultured with *t10c12CLA* and transfected with a luciferase plasmid containing the murine SREBP-1c promoter with mutated LXRE demonstrated a possible role for LXR in mediating the CLA response, but SREBP-1 was the predominant regulator of the SREBP-1c promoter in that experiment (Harvatine et al., 2014). Overall, in vivo and in vitro studies of CLA- and diet-induced MFD offer little or no support for a major role of LXR and PPAR γ in the regulation of milk fat synthesis in dairy cows (Oliveira et al., 2013; Harvatine et al., 2014).

To further investigate the mechanism of the inhibitory effect of *t10c12CLA* on SRE and SRE complex activation, we examined the role of the one or more double bonds in mediating the inhibition. As shown in Figure 2A and B, *t10 18:1* and *c12 18:1* increased SRE and SRE complex reporter activities compared with *t10c12CLA* ($P < 0.05$). Similar results were observed for *c9*

Figure 2. The double-bond structure of *t10c12CLA* on activities of reporters containing (A) sterol response element (SRE) and (B) SRE complex. Bovine mammary epithelial (MAC-T) cells were treated with *trans*-10,*cis*-12 CLA (*t10c12CLA*), *trans*-10 (*t10*) or *cis*-12 (*c12*) 18:1 at 100 μ M, followed by cotransfection with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 μ g/well) containing SRE or SRE complex in the reporter. Bovine serum albumin was used as a negative control and *cis*-9 (*c9*) and *trans*-11 (*t11*) 18:1 were used as FA conformational controls. After 24 h of transfection, cells were lysed and luciferase activities were measured. Luciferase activity was calculated by the ratio of firefly luciferase activity to renilla luciferase activity. Each experiment was repeated 3 times. Data (means $\pm$ SE) were normalized to the BSA control. *Different from BSA, $P < 0.05$; †different from *t10c12CLA*, $P < 0.05$.

18:1 and *t11* 18:1 controls. Moreover, neither *t10* 18:1 nor *c12* 18:1 decreased reporter activities when compared with BSA control. Instead, *t10* 18:1 significantly ($P < 0.05$) increased SRE reporter activity as shown in Figure 2A. These results indicate that the *t10* and *c12* double bonds are required to be present in a conjugated arrangement to inhibit SREBP-1 activation. The role of *t10* 18:1 in MFD has been controversial. Because of increased milk-fat *trans* 18:1 FA concentrations during MFD, these *trans* FA have been hypothesized to

cause MFD (Piperova et al., 2000). However, in vivo studies infusing physiological concentrations of different *trans* 18:1 isomers (*trans*-9, *trans*-11, and *trans*-12 18:1; see review by Harvatine et al., 2009) including *trans*-10 18:1 (Lock et al., 2007) found these did not cause MFD in dairy cows. As more direct evidence, our results offer no support for *t10* 18:1 playing a role in inhibiting lipogenesis through SREBP-1 in bovine mammary epithelial cells.

In conclusion, we confirmed the inhibitory effect of *t10c12CLA* on SRE-luciferase reporter activation but not on PPRE or LXRE in cultured bovine mammary epithelial cells. Both *t10* and *c12* double bonds have to be present to exhibit the inhibitory effect on SRE reporters. This study provides some direct evidence and insights into the transcriptional regulation of fat synthesis by SREBP-1 during *t10c12CLA*-induced lipogenic inhibition in bovine mammary epithelial cells.

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