Regulatory factors of milk fat synthesis in dairy cows

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ABSTRACT

The objective of these studies was to investigate the milk fat synthesis regulation by transcription factors. In the first study, bovine mammary epithelial (MAC-T) cells were treated with sterol regulatory element binding protein-1 (SREBP-1) specific siRNA. The mRNA and protein expression of SREBP-1 were decreased by more than 90% by siRNA. Fatty acid (FA) synthesis, uptake, and selected lipogenic enzyme expression were reduced in cells treated with SREBP-1 siRNA. Therefore, SREBP-1 plays an important role in integrated regulation of lipid synthesis in MAC-T cells through regulation of key enzymes. In the second study, MAC-T cells treated with hormones or FA were transfected with luciferase reporter constructs containing response elements for SREBP-1, peroxisome proliferator-activated receptor γ (PPARγ), or liver X receptor (LXR). The activation of PPARγ and SREBP-1 were stimulated by insulin and insulin combined with leptin, respectively. *Trans*-10, *cis*-12 conjugated linoleic acid (CLA) inhibited SREBP-1 activation, and this inhibition was not attenuated by insulin and leptin. Neither *trans*-10 nor *cis*-12 double bond inhibited SREBP-1 activation. Taken together, *trans*-10 and *cis*-12 double bonds need to be conjugated in CLA to reduce SREBP-1 activation and this inhibition cannot be overcome by insulin and leptin combination in MAC-T cells. In the third study, lactating dairy cows were intravenously infused with 0.625 g/h *trans*-10, *cis*-12 CLA for 14 h. We confirmed the appearance of *trans*-10, *cis*-12 CLA in the milk of CLA treated cows. Milk and component yield were not affected by the CLA treatment. The desaturation of stearic

acid was reduced by CLA. The mRNA and protein expression of transcription factors or lipogenic enzymes were not affected by *trans*-10, *cis*-12 CLA. DNA-binding activities for PPARγ and LXR and the activation of SREBP-1 to its mature form were not changed by the treatment. The infusion time in this study was probably too short to induce any changes in transcription factors and lipogenic enzymes. We confirmed DNA-binding activities of PPARγ and LXR in bovine mammary gland. Overall, a prominent role for SREBP-1 in mammary epithelial cell lipid synthetic pathways was described and regulation of transcription factor activation by *trans*-10, *cis*-12 CLA was specific to SREBP-1.

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LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACSL	acyl-CoA synthetase long-chain family
ACSS	acyl-CoA synthetase short-chain family
ADPH	adipophilin
AGPAT	acylglycerol-3-phosphate acyltransferase
bHLHLZ	basic helix-loop-helix leucine zipper
BTN	butyrophilin
BTN1A1	butyrophilin subfamily 1 member A1
CD36	cluster of differentiation 36
CLA	conjugated linoleic acid
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
EIF3K	eukaryotic translation initiation factor 3 subunit K
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FA	fatty acid
FABP3	fatty acid binding protein
FAS	fatty acid synthase
GPAM	mitochondrial glycerol-3-phosphate acyltransferase
GPAT	glycerol-3-phosphate acyltransferase
IDH1	isocitrate dehydrogenase

Insig	insulin induced gene
LPA	lysophosphatidic acid
LPIN	lipin
LPL	lipoprotein lipase
LXR	liver X receptor
LXRE	liver X receptor response element
MFD	milk fat depression
NADPH	nicotinamide adenine dinucleotide phosphate
PA	phosphatidic acid
PAS	periodic acid/Schiffn
PLIN	perilipin
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RPS15	ribosomal protein S15
RXR	retinoic acid receptor
S14	spot 14
S1P	site 1 protease
S2P	site 2 protease
SCAP	SREBP cleavage activating protein
SCD	stearoyl-CoA desaturase
siRNA	small interfering RNA
SRE	sterol response element
SREBP	sterol regulatory element binding protein

TAG	triacylglycerol
XDH	xanthine oxidoreductase

Chapter 1

General Introduction

As a major component in milk, milk fat plays an important role in supplying energy and accounts for many physical properties and manufacturing characteristics of milk and milk products (Bauman and Griinari, 2001). Milk fat is mainly composed of triacylglycerol (**TAG**) with 3 fatty acids (**FA**) esterified into the glycerol-3-phosphate backbone. Fatty acids are classified according to carbon chain length and saturation. Based on chain length, FA are grouped as short-chain FA (4-8 carbons), medium-chain FA (10-14 carbons), and long-chain FA (16 and more carbons). Fatty acids are also classified by desaturation, including saturated FA (no double bond), monounsaturated FA (one double bond), and polyunsaturated FA (more than one double bond). There are two sources of FA for milk fat synthesis, the *de novo* FA synthesis in mammary epithelial cells and preformed FA uptake from blood circulation derived from either diet or mobilized body fat (Barber et al., 1997). To synthesize milk fat, many enzymatic activities are involved in the pathways, including FA activation, transport, desaturation, TAG synthesis, milk fat globule formation and secretion (Clegg et al., 2001). Evidence suggests that several transcription factors are associated with milk fat synthesis, including sterol regulatory element binding protein-1 (**SREBP-1**), peroxisome proliferator-activated receptor γ (**PPARγ**), and liver X receptor (**LXR**) (Kadegowda et al., 2009; McFadden and Corl, 2010; Rudolph et al., 2010). However, the specific roles of each transcription factor in regulating milk fat synthesis are still unknown.

The fat content in milk may be markedly influenced by diet. A low fat syndrome, referred to as milk fat depression (**MFD**), is caused by diets containing large amounts of readily

fermentable carbohydrates and reduced amounts of roughage, and diets supplemented with polyunsaturated oils (Bauman and Griinari, 2003). To explain the mechanism of MFD, numerous theories have been postulated and examined over the last century. A widely accepted theory is the biohydrogenation theory based on the concept that under certain dietary conditions, the biohydrogenation pathways of unsaturated FA in the rumen are altered to produce some unique FA intermediates, mainly conjugated linoleic acids (**CLA**), which cause reduced milk fat synthesis (Bauman and Griinari, 2001). Based on this knowledge, research on MFD at molecular levels has been conducted to reveal which and how FA intermediates affect milk fat synthesis.

Chouinard et al. (1999a) observed a reduction in milk fat content and yield in cows abomasally infused with a CLA mixture mainly containing *cis*-8, *trans*-10 CLA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, and *cis*-11, *trans*-13 CLA, demonstrating the inhibitory effect of CLA on milk fat production in dairy cows. To identify the specific CLA isomer that causes MFD, Baumgard et al. (2000) treated lactating dairy cows with either *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA by abomasal infusion and observed decreased milk fat percentage and yield only in the *trans*-10, *cis*-12 CLA treatment, demonstrating that *trans*-10, *cis*-12 CLA is an isomer responsible for MFD.

The mRNA expression of lipogenic enzymes involved in *de novo* pathway (acetyl-CoA carboxylase (**ACC**) and fatty acid synthase (**FAS**)) and TAG synthesis (glycerol phosphate acyltransferase (**GPAT**) and acylglycerol phosphate acyltransferase (**AGPAT**)) was decreased in dairy cows with MFD induced by a high concentrate/low forage diet, indicating transcription as a potential point of regulation for milk fat synthesis (Peterson et al., 2003). Additionally, there is evidence showing reduced mRNA abundance of SREBP-1 in cows during MFD caused by a low forage/high oil diet, indicating a possible regulatory role of SREBP-1 in milk fat synthesis

(Harvatine and Bauman, 2006). As down-stream targets, FAS and stearoyl-CoA desaturase 1 (**SCD1**) are shown to have sterol regulatory elements (**SRE**), response elements for SREBP-1, in their promoters (Peterson et al., 2004). Therefore, SREBP-1 might play a critical role in regulating milk fat synthesis in cows with MFD. In addition to SREBP-1, PPARγ and LXR might also play important roles in milk fat synthesis regulation. Kadegowda et al. (2009) showed induced lipogenic gene expression in bovine mammary epithelial cells by the PPARγ agonist, rosiglitazone, indicating a possible role of PPARγ in regulating milk fat synthesis. Liver X receptor activation regulates SREBP-1 in bovine mammary epithelial cells, revealing the possible function of LXR in regulating milk fat synthesis in dairy cows (McFadden and Corl, 2010). However, the specific roles of these transcription factors in milk fat regulation have not been determined. Therefore, we hypothesized that the lipogenic enzymes responsible for milk fat synthesis are transcriptionally regulated by these transcription factors and transcriptional regulation of lipogenic enzymes is one of the mechanisms for milk fat reduction during MFD.

The objectives of the following research were 1) to determine the specific role of SREBP-1 in regulating lipid synthesis in bovine mammary epithelial cells; 2) to examine the effect of FA and hormones on the activation of luciferase reporters containing response elements for SREBP-1, PPARγ and LXR; and 3) to reveal the effect of short-term *trans*-10, *cis*-12 CLA infusion on transcription factor activation in the mammary gland of lactating dairy cows.

Chapter 2

Review of literature

MILK FAT SYNTHESIS

The content of lipid present in cow milk is usually about 4% (Garton, 1963). In milk fat, the most predominant lipid class (more than 95%) is TAG, followed by approximately 2% of diacylglycerol (**DAG**), while other lipids include small amounts of phospholipids and cholesterol, about 1 and 0.5%, respectively, and a very small fraction of free FA (about 0.1%) (Jensen and Newberg, 1995). In addition, trace amounts of ether lipids, hydrocarbons, fat-soluble vitamins, flavor compounds and compounds introduced by the feed are present in milk fat (Parodi, 2004).

As a major component in milk, milk fat contains approximately 400 different FA, making it the most complicated natural fat (Mansson, 2008). Due to hydrogenation of dietary polyunsaturated FA by bacteria in the rumen, around 70% of FA by weight in milk fat are saturated, with palmitic acid (16:0) the most abundant, accounting for approximately 30% by weight of the total FA, followed by myristic acid (14:0) and stearic acid (18:0) (MacGibbon and Taylor, 2006). In addition to saturated FA, unsaturated FA containing one or more double bonds are present in milk fat as well. Approximately 25% of the milk FA are monounsaturated and oleic acid (18:1) is the most abundant. Unlike monounsaturated FA, polyunsaturated FA only account for a small portion of total FA in milk, with linoleic acid (18:2) and α-linolenic acid (18:3) the most abundant. With one or more *trans* double bonds, *trans* FA comprise approximately 2.7% of the FA in milk and the main *trans* 18:1 isomer is vaccenic acid (*trans*-11 18:1) (MacGibbon and Taylor, 2006; Precht and Molkentin, 1995). The presence of CLA is

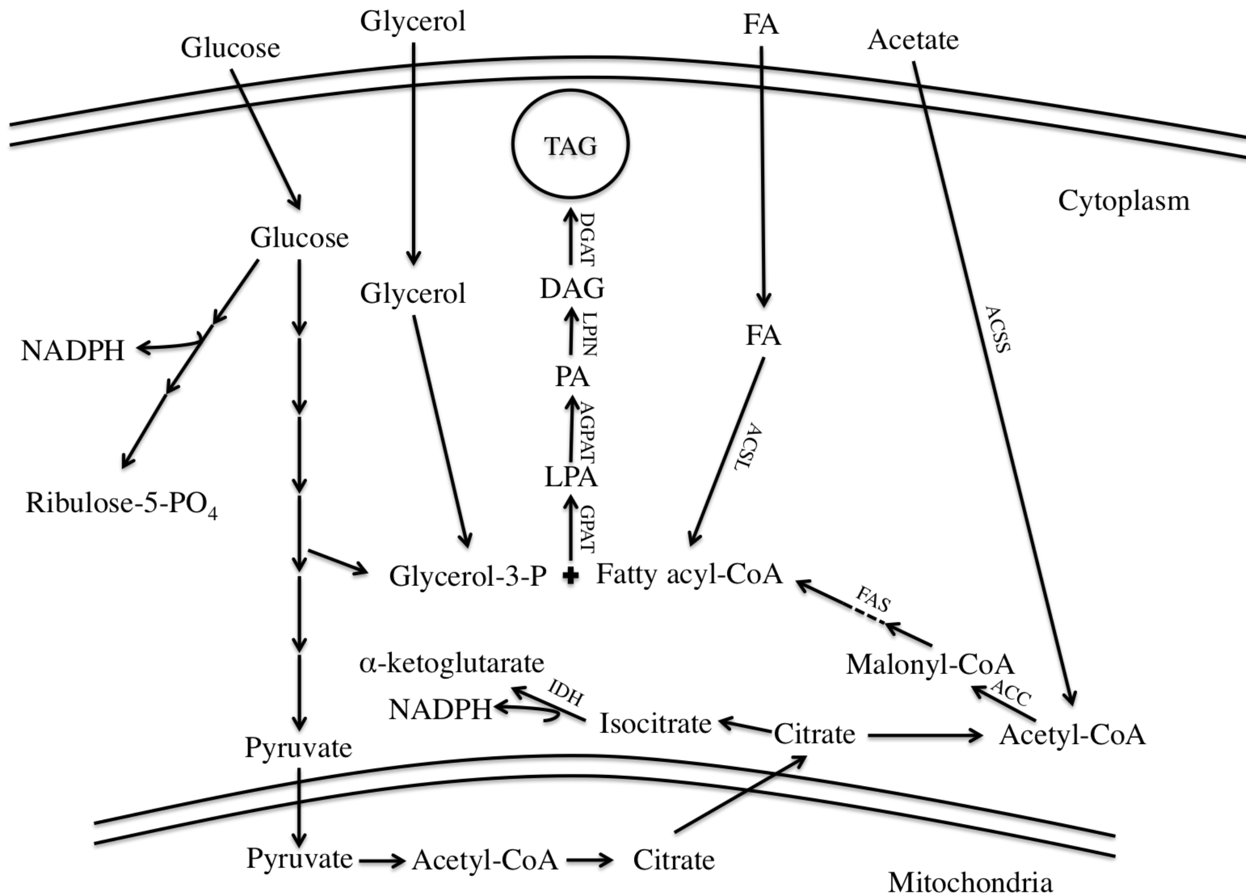
confirmed as well, with *cis*-9, *trans*-11 isomer the most predominant (75-90% of total CLA) (Precht and Molkentin, 1995).

The FA used for milk fat synthesis are derived from two sources, *de novo* synthesis in the mammary epithelial cells and preformed FA originating from the diet or body fat mobilization (Dils, 1986; Neville and Picciano, 1997). Short-chain (4 to 8 carbons) and medium-chain (10-14 carbons) FA are almost exclusively *de novo* synthesized in the mammary gland; long-chain FA (> 16 carbons) are derived from the uptake of circulation; while FA with 16 carbons are equally from both sources (Bauman and Griinari, 2003). The pathways for milk fat synthesis are shown in Figure 2.1.

De novo fatty acid synthesis

In ruminants, the substrates for *de novo* FA synthesis in mammary epithelial cells are acetate produced by rumen fermentation of carbohydrates and β-hydroxybutyrate produced by the rumen epithelium from absorbed butyrate (Mansbridge and Blake, 1997). To be activated, acetate is converted to acetyl-CoA by acyl-CoA synthetase short-chain family (**ACSS**). The conversion of acetyl-CoA to malonyl-CoA, catalyzed by ACC, is the rate-limiting step in *de novo* FA synthesis (Ha and Kim, 1994). Initially using acetyl-CoA as the substrate, FAS catalyzes a sequence of reactions, adding two carbons derived from malonyl-CoA to a growing fatty acyl chain each time (Smith, 1994). Each cycle requires two molecules of reducing equivalents (nicotinamide adenine dinucleotide phosphate, **NADPH**), generated from the pentose phosphate cycle and oxidation of isocitrate by isocitrate dehydrogenase (**IDH**) (Vernon and Flint, 1983). Transacylase is involved in the termination of fatty acyl synthesis up to 16 carbons (Knudsen and Grunnet, 1982).

Figure 2.1 Milk fat synthesis pathways



For *de novo* fatty acid (FA) synthesis, acetate is activated by acyl-CoA synthetase short-chain family (ACSS) to synthesize acetyl-CoA, which is subsequently converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). The enzyme fatty acid synthase (FAS) is responsible for the fatty acyl chain growth to produce fatty acyl-CoA up to 16 carbons. The reducing equivalent needed for *de novo* FA synthesis, nicotinamide adenine dinucleotide phosphate (NADPH), is derived from either the pentose phosphate cycle where ribulose-5-PO_4 is produced from glucose or the oxidation of isocitrate catalyzed by isocitrate dehydrogenase (IDH). Preformed FA taken up from blood are activated by acyl-CoA synthetase long-chain family (ACSL) to form fatty acyl-CoA ($\geq$ 16 carbons). Together with the fatty acyl-CoA from *de novo* pathway, they are esterified to glycerol-3-phosphate to produce triacylglycerol (TAG). The first fatty acyl-CoA is esterified into glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA). Lysophosphatidic acid is converted to phosphatidic acid (PA) by adding another fatty acyl-CoA to the sn-2 position, catalyzed by acylglycerol-3-phosphate acyltransferase (AGPAT). The phosphate group in PA is then removed by the phosphatidic acid phosphohydrolase enzyme lipin (LPIN) to produce diacylglycerol (DAG). Finally, TAG is formed by esterifying the third fatty acyl-CoA, catalyzed by diacylglycerol acyltransferase (DGAT).

Preformed fatty acid uptake

Long-chain FA taken up by the mammary gland and used for milk fat synthesis are imported from plasma, where they are released from circulating lipoproteins by lipoprotein lipase (**LPL**) or nonesterified FA bound to albumin that originate from the digestive tract or body fat mobilization (Clegg et al., 2001). In ruminants, preformed FA in milk fat taken up from circulation are predominantly from dietary and microbial FA absorbed from the digestive tract. Typically, there is less than 10% FA in milk fat derived from mobilization of body fat. However, when cows are in a negative energy balance, the contribution from mobilized FA increases (Bauman and Griinari, 2003).

The mechanism by which FA traverse the capillary endothelium and interstitial space to reach the alveolar cell is not known. There is evidence showing that the membrane transport of long-chain FA is a facilitated process (Abumrad et al., 1998). However, this process has not been studied in the mammary alveolar cell. Cluster of differentiation 36 (**CD36**) and fatty acid binding protein 3 (**FABP3**) might play a role in FA uptake and transport, because CD36 is a homolog of fatty acid translocator, a putative FA transporter in rat adipocytes, and FABP3 is the most abundant FABP isomer in bovine mammary gland (Abumrad et al., 1993; Bionaz and Loor, 2008a; Clegg et al., 2001; Harmon et al., 1991). Fatty acids entered mammary epithelial cells are activated by the acyl-CoA synthetase long-chain family (**ACSL**) enzymes, and channeled toward TAG synthesis.

Glycerol-3-phosphate pathway

Triacylglycerols are synthesized in the endoplasmic reticulum (**ER**) of mammary epithelial cells. Fatty acyl-CoAs from the *de novo* pathway and preformed FA are esterified to the glycerol-3-phosphate backbone to produce TAG. Lysophosphatidic acid (**LPA**) is formed by

adding a fatty acyl-CoA to sn-1 position of glycerol-3-phosphate, catalyzed by the enzyme GPAT. A second fatty acyl-CoA is incorporated at the sn-2 position by AGPAT to synthesize phosphatidic acid (**PA**). The phosphate group is removed by the PA phosphohydrolase enzyme lipin (**LPIN**), converting PA to DAG. Finally, TAG is synthesized by esterifying another fatty acyl-CoA to the sn-3 position of the glycerol backbone.

Fatty acids are not esterified randomly to the sn-1, -2, and -3 positions of glycerol backbone. Approximately 56-62% of FA at sn-1 and -2 positions are medium- and long-chain FA with 10 to 18 carbons. Additionally, about 24% of FA at the sn-1 position is oleic acid. Short-chain FA and oleic acid account for a large proportion of FA at sn-3 position, 44 and 27%, respectively (Bernard et al., 2008). The distribution of FA is dependent on the distinct binding affinities of the acyltransferase enzymes for substrate FA and results in this disproportionate esterification profile.

Milk fat secretion

Milk fat is secreted as milk fat globules, which are formed in the ER membrane by the incorporation of newly synthesized TAG. Milk fat globules are transported to the apical plasma membrane and released by the cell (Keenan and Mather, 2006). Lipid droplets are formed in the intermediate space of the ER bilayer membrane and released into cytosol coated with lipids and proteins from the cytoplasmic half of the ER membrane (Zaczek and Keenan, 1990). In the cytoplasm, some milk lipid droplets coalesce into larger droplets before and during secretion (Stemberger and Patton, 1981). These, in turn, migrate toward the apical plasma membrane of mammary epithelial cells probably in association with apical-basally oriented microtubules (Welte, 2009). Milk fat droplets become enveloped in plasma membrane and ultimately pinch off from the cell, bearing a continuous coat of specialized plasma membrane called the milk fat

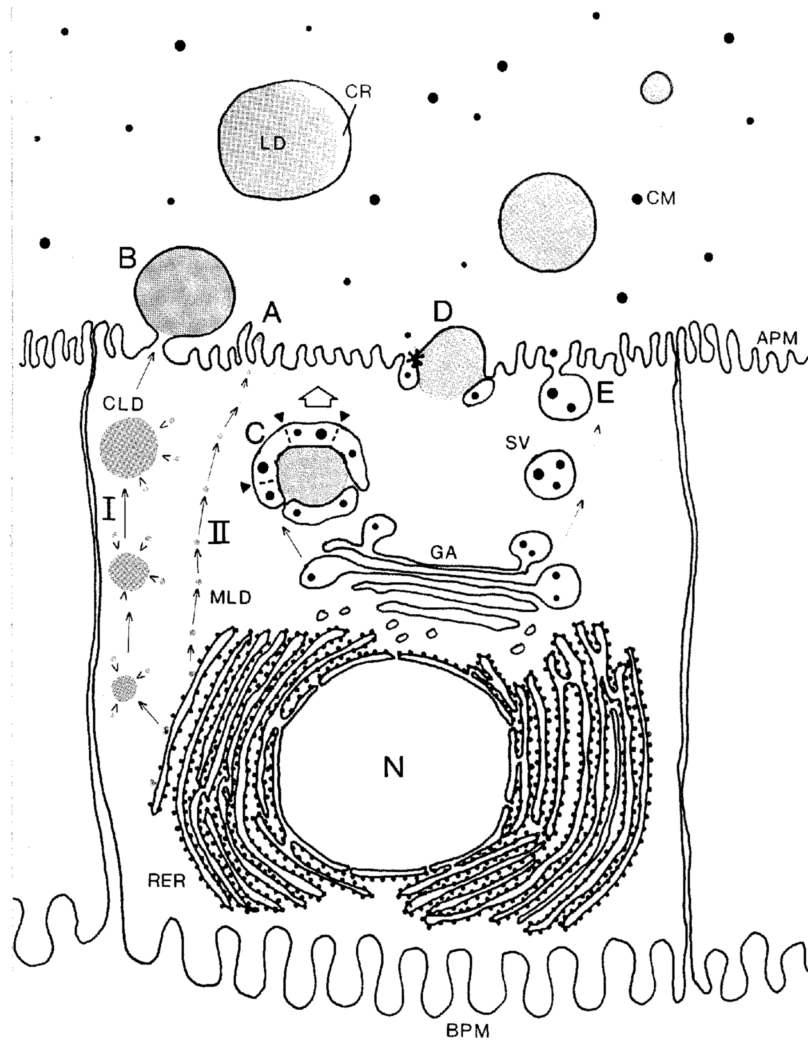
globule membrane. Cytoplasmic remnants are trapped between the outer membrane layer and the lipid globule in some secreted fat droplets. Alternatively, many milk fat droplets progress to the apical plasma membrane directly without further accretion in size. Under certain circumstances, fat droplets are surrounded by secretory vesicles and progressively fuse with each other to form cytoplasmic vacuoles. These vacuoles transit to the apical membrane and the contents are released by exocytosis. Caseins are processed through this pathway and secreted with the aqueous phase of milk or by exocytosis from secretory vesicles at the apical plasma membrane (Mather and Keenan, 1998). The secretion pathways for milk fat are illustrated in Figure 2.2.

The protein coat on the milk fat globule membrane comprises mainly butyrophilin (**BTN**), xanthine oxidoreductase (**XDH**), adipophilin (**ADPH**), mucin 1, CD36, Periodic acid/Schiff (**PAS**) 6/7, PAS III, and FABP (Mather, 2000). There is direct evidence that XDH and BTN are essential for milk fat globule secretion (Ogg et al., 2004; Vorbach et al., 2002). Adipophilin is required for lipid droplet maturation in mammary alveolar cells and plays an important role for alveolar differentiation and milk lipid secretion (Chong et al., 2011; Russell et al., 2011).

MILK FAT DEPRESSION

The concept of MFD has been proposed in 1970's (Bauman and Griinari, 2001). Milk fat depression is a low-fat syndrome in which milk fat content is reduced while other components in milk and milk yield remain unchanged. As the most variable component in milk, milk fat is affected by many factors, including genetics, stage of lactation, and feed (Palmquist et al., 1993). Diet-induced MFD has been the interest of many research scientists. Diets causing MFD can be divided into two groups. One group is the diets containing large amounts of readily digestible carbohydrates or reduced amounts of roughage; the second group comprises diets supplemented with unsaturated fat, such as fish oil and plant oil (Bauman and Griinari, 2001).

Figure 2.2 Pathways for milk fat globule transit and secretion from mammary epithelial cells



The nucleus (N) in the basal plasma membrane (BPM) is surrounded by the rough endoplasmic reticulum (RER) where milk lipid droplets (LD) are formed. Pathway I (mechanism B): These droplets fuse with each other or cytoplasmic lipid droplets (CLD) as they are transported to the apical plasma membrane (APM). Cytoplasmic remnants (CR) are trapped between the outer membrane and the lipid globule in some secreted LD. Pathway II (mechanism A): Some microlipid droplets (MLD) transit to the APM directly without fusing with other molecules. Mechanism C: Fat droplets are secreted in secretory vesicles (SV) produced by Golgi apparatus (GA) surround CLD and progressively form vacuoles, which are transported to the apical surface. Mechanism D: A combination of both apical and secretory vesicle routes may be possible. Mechanism E: Lipid droplets are secreted with by simple exocytosis from SV. Reproduced from Mather and Keenan (1998).

Peterson et al. (2003) observed a reduction in milk fat percentage (25%) and yield (27%) in cows fed a high concentrate/low forage diet, while feed intake, milk yield, and protein and lactose production were not changed. In the same study, FA from *de novo* synthetic pathway and uptake from circulation were reduced to a similar extent on molar basis. The appearance of *trans*-10, *cis*-12 CLA in milk fat was also characterized in cows with diet-induced MFD. A similar milk fat yield decrease (38%) was reported in cows fed a low forage/high oil diet when compared to the control cows (Harvatine and Bauman, 2006). In addition, FA in the study were divided into 3 groups based on the chain length, > 16 carbons, 16 carbons, and < 16 carbons, and the FA in all categories were reduced in cows fed the MFD-inducing diet. Therefore, milk fat is markedly decreased during diet-induced MFD, and FA from both *de novo* pathway and circulation were reduced. To explain this low-fat syndrome, studies have been done to reveal the mechanisms associated with MFD.

Over the past century, many theories have been proposed to explain MFD. The theories for diet-induced MFD are divided into two categories, including those based on the hypothesis that decreased milk fat yield during MFD is due to an inadequate supply of lipogenic precursors for milk fat synthesis in mammary gland, and theories that attribute the decline in milk fat content to a direct inhibition of one or more steps in milk fat biosynthetic pathways. The representative theory for the former category is the glucogenic-insulin theory, while examples in the latter category include *trans* FA theory and the biohydrogenation theory (Bauman and Griinari, 2001).

Glucogenic-insulin theory

The glucogenic-insulin theory is proposed based on different tissue responses to insulin, which acutely stimulates lipogenesis and inhibits lipolysis in adipose tissue, but has little effect

on ruminant mammary gland because it is not responsive to changes of circulating insulin (Komatsu et al., 2005; Zhao and Keating, 2007). This provides the basis for the glucogenic-insulin theory as the cause of MFD, which is induced by diets high in readily digestible carbohydrates and low in fiber. According to the glucogenic-insulin theory, the MFD-inducing diets result in increased production of propionate and glucose, which stimulate pancreatic release of insulin. Elevated insulin enhances the uptake of precursors for lipogenesis by adipose tissue and limits adipose lipolysis, so that FA released by adipose tissue are decreased and the mammary gland is deprived of milk fat precursors (Jenny et al., 1974). Overall, these changes would represent a preferential channeling of nutrients to adipose tissue, resulting in a shortage of lipogenic precursors for milk fat synthesis in the mammary gland.

To verify the glucogenic-insulin theory, the role of insulin in milk fat synthesis has been examined using a hyperinsulinemic-euglycemic clamp to avoid hypoglycemia and counter-regulatory changes in glucose homeostasis. In support of the lack of insulin sensitivity of the mammary gland, milk yield was not changed; milk fat percentage and yield remained unchanged during the insulin clamp, offering no support for the glucogenic-insulin theory of MFD (McGuire et al., 1995). In addition to the constant rate of milk fat synthesis during the clamp infusion, the changes in milk FA composition were minor and the balance between *de novo* and preformed FA shifted slightly towards *de novo* (Griinari et al., 1997). These results are different from the observations from diet-induced MFD, in which milk fat was decreased and the FA composition was markedly altered because the decline was greatest for *de novo* synthesized FA (Bauman and Griinari, 2001). Taken together, there is no evidence showing insulin plays a role in regulating milk fat synthesis in dairy cows during MFD.

***Trans* fatty acid theory**

Trans FA are formed as intermediates during the biohydrogenation of dietary unsaturated FA by bacteria in the rumen, with *trans*-11 18:1 being the predominant *trans* isomer (Griinari and Bauman, 1999). The involvement of *trans* FA in the development of MFD has been most often considered with diets containing plant or fish oils. Increases in *trans* 18:1 FA content of milk fat were observed when cows were fed a low fiber/high corn oil diet and a classical high concentrate/low forage diet (Griinari et al., 1998). Milk fat production was decreased linearly with the amount of *trans* 18:1 FA flowing into the duodenum and the concentration of *trans* 18:1 in milk (Wonsil et al., 1994). The role of *trans* 18:1 FA in MFD was further examined using abomasal infusion, showing that *trans* FA depressed milk fat percentage and yield (Gaynor et al., 1994).

The involvement of *trans* FA in reducing milk fat synthesis during MFD has been examined more directly using partially hydrogenated vegetable oil as a source of *trans* 18:1 FA. Most of those studies did not report the positional distribution of *trans* FA and therefore were insufficient to reveal the roles of specific *trans* FA in the reduction of milk fat synthesis. Thus, studies using pure *trans* FA are needed to further determine whether they play a role associated with MFD. Rindsig and Schultz (1974) reported that milk fat yield or percentage were not affected when cows were infused with *trans*-9 18:1 isomer. Additionally, abomasal infusion of pure *trans*-11 18:1 and *trans*-12 18:1 did not change the content of milk fat (Griinari et al., 2000). These studies indicate that the *trans* 18:1 FA investigated do not influence milk fat synthesis.

The identification of specific *trans* FA isomers, if any, related to MFD is essential to reveal the mechanism for MFD. *Trans*-10 18:1 content in milk was elevated in cows with MFD induced by a low fiber diet (Griinari et al., 1998). However, some other studies do not confirm

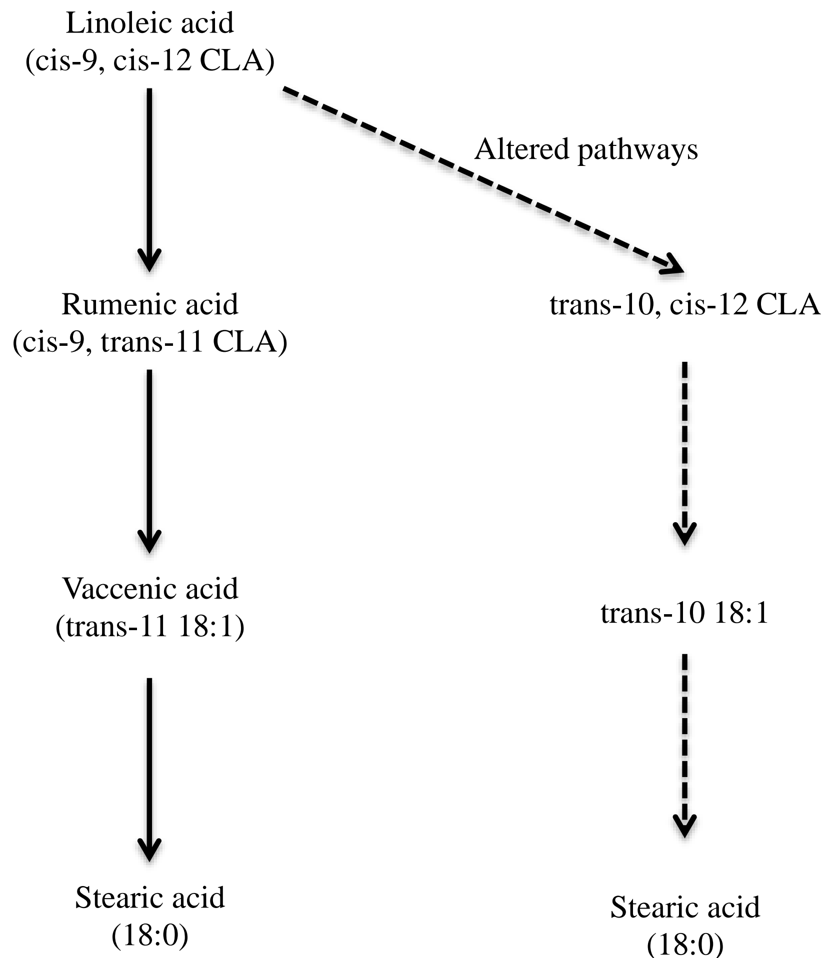
the involvement of *trans*-10 18:1 in diet-induced MFD. For instance, Lock et al. (2007) demonstrated that *trans*-10 18:1 was not a cause for MFD due to the fact that milk fat yield was not affected during abomasal infusion of *trans*-10 18:1 for 4 d, providing 42.6 g/d *trans*-10 18:1.

Biohydrogenation theory

The *trans* FA theory is based on the load of total *trans* FA. As presented above, there is no direct evidence verifying this theory. Therefore, this theory needs to be modified to better explain experimental results. The new theory is referred to as the biohydrogenation theory, based on the concept that, under certain dietary conditions, the pathways of rumen biohydrogenation are altered to produce unique FA intermediates that are potent inhibitors of milk fat synthesis (Bauman and Griinari, 2001). When there are large amounts of carbohydrate or small amounts of fiber, the environment of rumen is changed and the biohydrogenation pathways are altered. Under these conditions, linoleic acid is converted to *trans*-10, *cis*-12 CLA instead of *cis*-9, *trans*-11 CLA. *Trans*-10, *cis*-12 CLA is further reduced to give *trans*-10 18:1. The biohydrogenation pathways are shown in Figure 2.3.

The effect of CLA on milk fat synthesis was confirmed by observing a marked reduction of milk fat yield in dairy cows receiving CLA abomasally (Chouinard et al., 1999a). To identify the specific CLA isomer involved in MFD, Baumgard et al. (2000) showed that *trans*-10, *cis*-12 CLA was the isomer responsible for inhibition of milk fat during MFD. The role of *trans*-10, *cis*-12 CLA in causing MFD was further confirmed by studies using abomasal infusion. Baumgard et al. (2001) showed a progressive reduction in milk fat synthesis when cows were abomasally infused with increasing amounts of *trans*-10, *cis*-12 CLA (0-14 g/d), and revealed that *de novo* synthesized FA were extensively reduced at the two high doses, consistent with what was observed in diet-induced MFD studies.

Figure 2.3 A summary of biohydrogenation pathways



Under normal conditions, linoleic acid in the rumen is converted to rumenic acid (*cis*-9, *trans*-11 CLA), which is used to synthesize vaccenic acid (*trans*-11 18:1). The final product of this pathway is stearic acid (18:0). However, when cows are fed with high concentrate/low forage diets, the biohydrogenation of unsaturated FA goes through an altered pathway. In the altered pathway, instead of rumenic acid, *trans*-10, *cis*-12 CLA is synthesized from linoleic acid. Then *trans*-10 18:1 is produced from *trans*-10, *cis*-12 CLA. The final product is the same as the classical biohydrogenation pathway. Modified from Bauman and Griinari (2003).

Considering the involvement of lipogenic enzymes in milk fat synthesis, the hypothesis is that *trans*-10, *cis*-12 CLA has inhibitory effect on these enzymes. The mRNA expression of enzymes involved in *de novo* pathway (ACC and FAS), FA desaturation (SCD1), FA trafficking (FABP), and TAG synthesis (GPAT and AGPAT) was decreased in both diet- and *trans*-10, *cis*-12 CLA infusion-induced MFD, indicating the inhibition of lipogenic enzymes by *trans*-10, *cis*-12 CLA during MFD (Baumgard et al., 2002; Baumgard et al., 2001; Peterson et al., 2003). Because mRNA is the product of transcription, the lipogenic enzymes might be regulated at transcriptional level. Therefore, transcription factors are possibly involved in the regulation of milk fat synthesis.

TRANSCRIPTION FACTORS

Transcription factors are a group of proteins that bind to short DNA sequences and influence the transcription of genes either positively or negatively. Essentially they determine whether a particular gene will be turned 'on' or 'off' in an organism. Transcription factors regulate transcription of the DNA by binding to DNA through the DNA binding domain and the DNA sequences binding to transcription factors are called response elements. There are also transcriptional activation/inhibition domains which have stimulatory or inhibitory effects on transcription (Harrison, 1991). Additionally, some transcription factors are activated by ligands that are specific to the transcription factor, for example steroid hormone nuclear receptors.

A variety of binding domains have been identified according to the structures. Some well-characterized DNA binding domains include the helix-loop-helix motif, the two cysteine-two histidine zinc finger, the multi-cysteine zinc finger, the Ets domain, and the basic DNA binding domain (Latchman, 1997). The last example is of particular interest because factors containing the basic DNA binding domain only bind to DNA when they form transcription factor

dimers. Therefore, factors containing the basic binding domain are further sub-grouped based on the nature of the dimerization motifs they contain. For some transcription factors, the helix-loop-helix motif mediates dimerization (Murre et al., 1994). In contrast, some other basic DNA binding domain-containing transcription factors undergo dimerization via the leucine zipper motif that contains a regular array of leucine residues (Landschulz et al., 1998).

To control the transcription of genes, transcription factors contain activation domains that interact either directly with specific components in the basal transcription complex, consisting of RNA polymerase II and various transcription factors, or indirectly with co-activator molecules that interact with the basal complex to stimulate transcription (Latchman, 1997). Although many transcription factors are identified as stimulatory factors, a variety of transcription factors act by inhibiting gene transcription. The inhibitory transcription factors act by either directly binding to DNA or interfering the action of activators (Latchman, 1997). Hence, the balance between binding to transcription activators and transcription repressors to the gene will determine its expression in any particular situation.

Sterol regulatory element binding protein

Sterol regulatory element binding proteins belong to the basic helix-loop-helix leucine zipper (**bHLHLZ**) family of transcription factors and contain three domains: 1) an NH2-terminal transcription factor domain of about 480 amino acids; 2) a middle hydrophobic region of about 80 amino acids containing two hydrophobic transmembrane segments; and 3) a COOH-terminal regulatory domain of approximately 590 amino acids (Brown and Goldstein, 1997). Three SREBP isomers have been identified, SREBP-1a, -1c, and -2 (Hua et al., 1993; Yokoyama et al., 1993). The two isoforms of SREBP-1, with distinct first exons, are transcribed from the same gene via alternative splicing. However, SREBP-2 is transcribed from a separate gene. Sterol

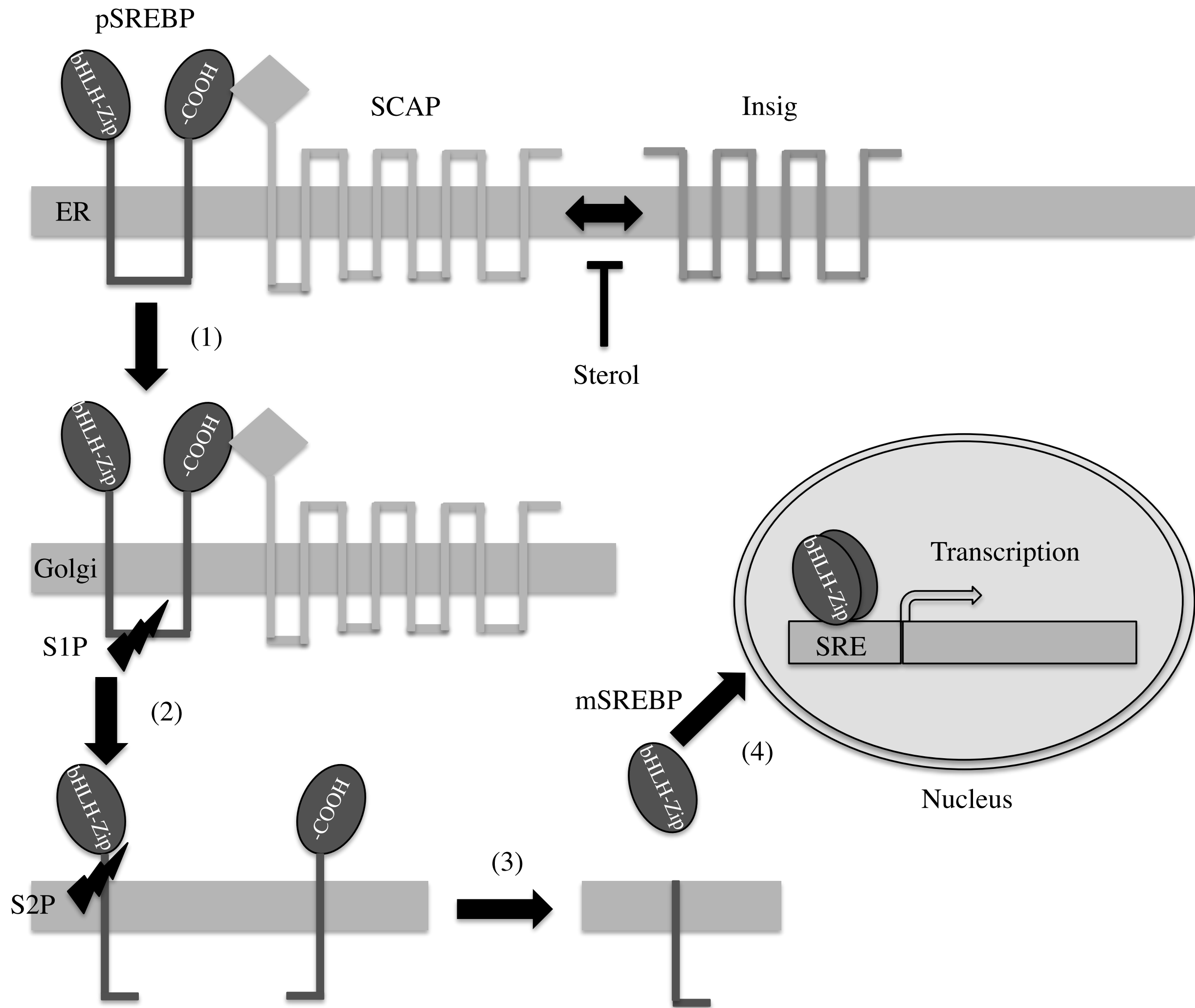
regulatory element binding protein-1c and 2 are involved in regulation of FA and cholesterol biosynthesis, respectively, whereas SREBP-1a is involved in both processes (Eberle et al., 2004).

Sterol regulatory element binding proteins are synthesized as inactive precursors and activated by serial proteolytic cleavages. Under certain conditions, such as presence of sterol, the SREBP cleavage activating protein (**SCAP**) associated with the SREBP protein undergoes a conformational change that promotes binding to the ER-resident protein, insulin induced gene (**Insig**). This retains the SREBP-SCAP complex in the ER compartment. When sterol is depleted, the binding between SREBP-SCAP complex and Insig is disrupted and SCAP is disassociated with Insig, escorting SREBP to the Golgi, where two sequential proteolytic cleavages occur, mediated by the site 1 (**S1P**) and site 2 (**S2P**) proteases. After cleavage, the N-terminus of SREBP (mature form) is released from the membrane and translocated into the nucleus, where the mature SREBP binds to SRE sequences (E-box, SRE, or related sites) in the promoters of target genes to activate transcription (Eberle et al., 2004). The activation of SREBP-1 is shown in Figure 2.4.

Peroxisome proliferator-activated receptor

Peroxisome proliferator-activated receptors, together with steroid, thyroid and retinoid hormone receptors, belong to the nuclear hormone receptor superfamily (Wahli et al., 1995). There are three subtypes of PPAR, including α, δ, and γ. In rodents, PPARδ is present at early stages of development (Wahli et al., 1995). Peroxisome proliferator-activated receptor α is highly expressed in tissues active in lipid metabolism (liver, kidney, white and brown adipose tissues, heart, skeletal muscle). Peroxisome proliferator-activated receptor γ is expressed at a high level in white adipose tissue, brown adipose tissue and spleen (Lemberger et al., 1996).

Figure 2.4 Sterol regulatory element binding protein activation by proteolytic cleavages



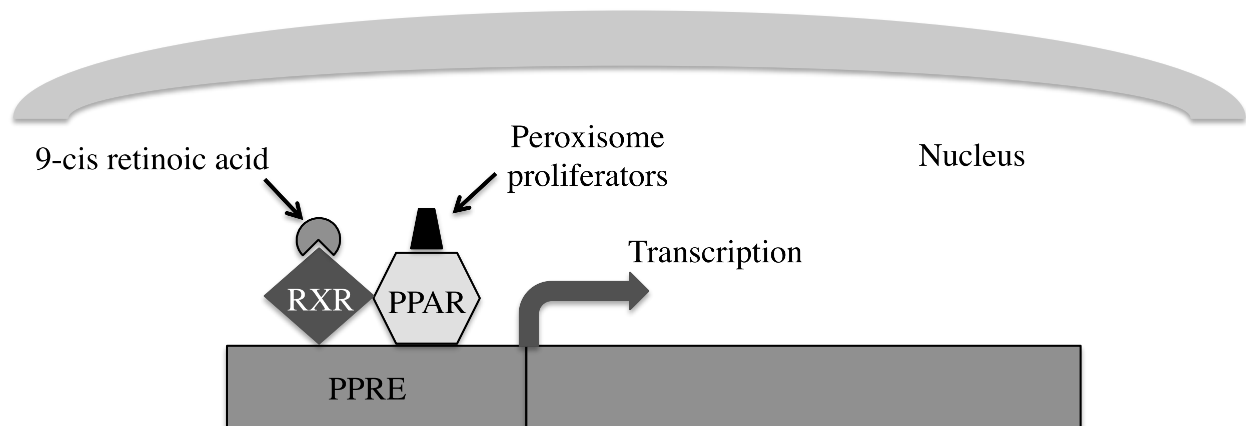
Sterol regulatory element binding proteins (SREBP) are synthesized as precursor proteins (pSREBP) bound to the membrane of the endoplasmic reticulum (ER). They are associated with SREBP cleavage activating protein (SCAP), which interacts with the insulin induced gene protein (Insig). When sterol is depleted, the interaction between SCAP and Insig decreases and (1) SCAP escorts SREBP to the Golgi apparatus. (2) Subsequently, the SREBP protein is proteolytically cleaved by site 1 protease (S1P), followed by (3) another cleavage by site 2 protease (S2P) to release the mature protein (mSREBP) containing the basic helix-loop-helix leucine zipper region (bHLH-Zipper). (4) Finally, mSREBP translocates to the nucleus where it binds to the target genes on sterol response elements (SRE) or E box (not shown) to induce gene expression. Modified from Eberle et al. (2004).

The three subtypes of PPAR regulate gene expression in various physiological processes. Peroxisome proliferator-activated receptor α plays a critical role in the regulation of cellular uptake, activation, and oxidation of FA (Martin et al., 1997; Schoonjans et al., 1995; Zhang et al., 1993). The role of PPARδ in alleviating insulin resistance, maintaining reproductive capacity, central nervous system development, and signal transduction in the nervous system have been confirmed (Granneman et al., 1998; Lim et al., 1999; Oliver et al., 2001; T. et al., 1999; Yang et al., 2006). Peroxisome proliferator-activated receptor γ regulates genes involved in adipocyte differentiation, FA synthesis, inflammation, and cancer (Chinetti et al., 1998; Kallen and Lazar, 1996; Sarraf et al., 1998; Tontonoz et al., 1994).

The activation of PPAR is induced by peroxisome proliferators, including hypolipidemic drugs, plasticizers and herbicides (Green, 1995). Furthermore, FA are PPAR ligands as well. Long-chain FA activate PPARα and its target genes involved in lipid metabolism and inflammatory responses in bovine kidney cells (Bionaz et al., 2012). In addition, the expression of PPARα and PPARδ in endometrium of dairy cows was decreased by dietary supplementation of long-chain omega-3-FA (MacLaren et al., 2011). So far, it appears that no one FA exclusively activates a given PPAR subtype. Fatty acids with a chain length of 10 or more carbons activate PPARs from several species, while those with shorter chains are much less active (Wahli et al., 1995).

During activation, PPAR heterodimerize with the 9-*cis* retinoic acid receptor (**RXR**), forming a complex that binds to a specific PPRE sequence in the promoters of target genes and therefore induce the transcription of those targets (Wahli et al., 1995). The mechanism for PPAR action is shown in Figure 2.5.

Figure 2.5 Peroxisome proliferator-activated receptor activation pathways



Peroxisome proliferators may bind to peroxisome proliferator-activated receptors (PPAR) directly and activate them. Peroxisome proliferator-activated receptors form heterodimers with 9-*cis* retinoic acid receptor (RXR) and bind to the peroxisome proliferator response element (PPRE) located upstream of target genes. The activated PPAR induce target gene expression in the nucleus.

Liver X receptor

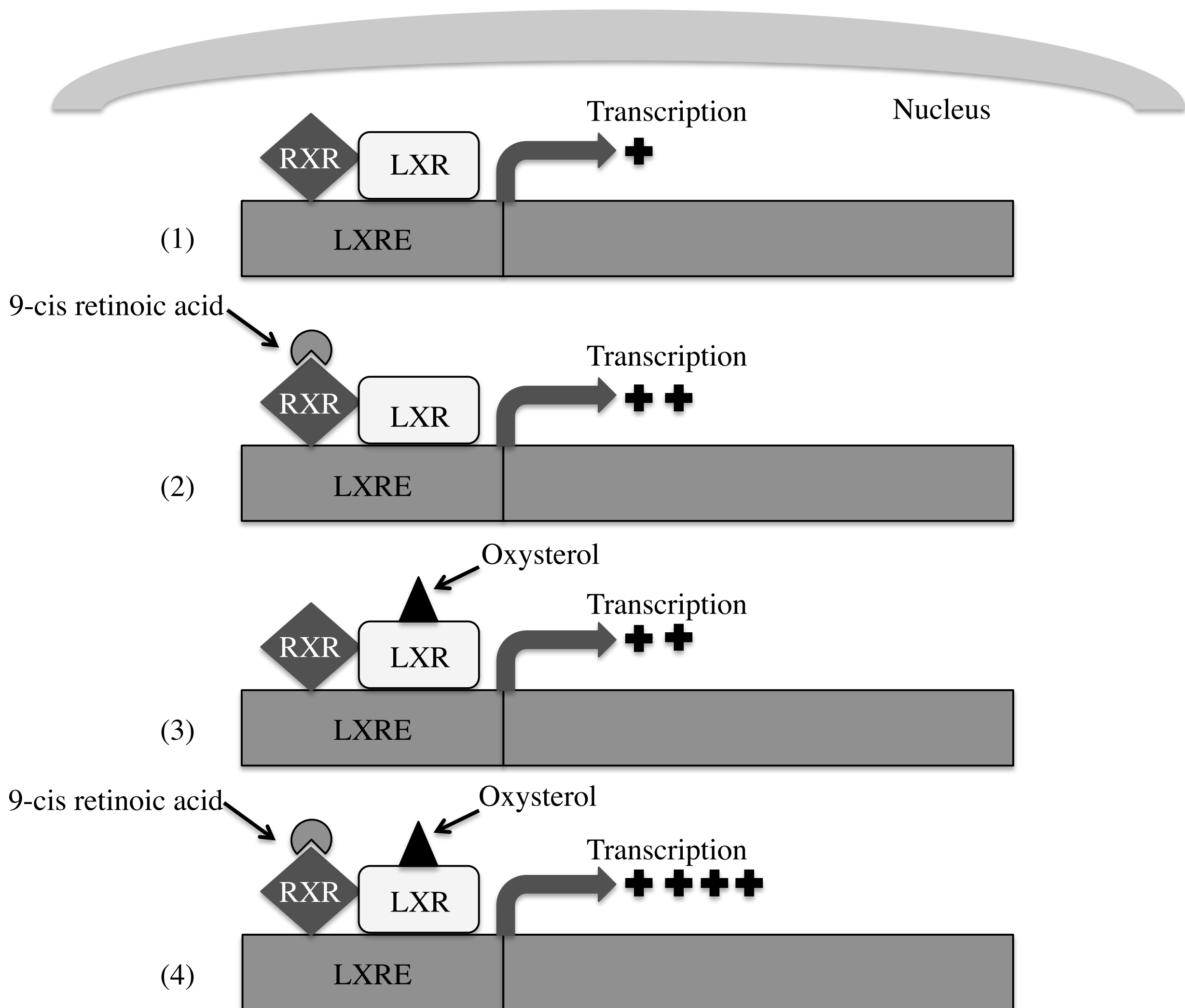
Liver X receptors are a family of nuclear receptors. Two members of the LXR family have been identified: LXRα and LXRβ. The two LXR proteins in human are closely related and share 77% amino acid identity in both their DNA- and ligand-binding domains. They are also highly conserved between humans and rodents (Peet et al., 1998). The tissue distribution of LXR isoforms differs. Liver X receptor α expresses predominantly in tissues active in lipid metabolism, for instance, liver, kidney, small intestine, spleen, adipose tissue, pituitary, and adrenals (Auboeuf et al., 1997; Willy et al., 1995). In contrast, the expression of LXRβ is more ubiquitous and it is present in almost every tissue examined, including liver and brain (Song et al., 1994). Studies have shown that LXRα is an indispensable regulator of cholesterol homeostasis. In contrast to LXRα, the role of LXRβ is still largely unknown. Liver X receptor α and β have a distinct set of target genes and thus have some non-overlapping roles.

Similar to PPAR, LXR also heterodimerize with RXR for function. The ligands for LXRs are identified as oxysterol, speicifically, a group of mono-oxidized derivatives of cholesterol and examples are 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 24(s), 25-epoxycholesterol (Janowski et al., 1996; Lehmann et al., 1997). In the absence of ligands, LXR-RXR dimer binds to the LXRE sequences in promoters of target genes and increases basal transcription of these genes. Either LXR ligand or RXR ligand addition leads to further conformational change of the dimer and substantially activates target gene expression. When both ligands are present, the LXR-RXR dimer induces gene transcription to the greatest extent (Peet et al., 1998). The activation pathways for LXR are shown in Figure 2.6.

Transcription factors in milk fat synthesis

As discussed, numerous enzymes are involved in milk fat synthesis. These enzymes are

Figure 2.6 Liver X receptor activation pathways



Liver X receptors (LXR) form heterodimers with 9-*cis* retinoic acid receptor (RXR) and bind to the LXR response elements (LXRE) in promoters of target genes. (1) In the absence of ligands, LXR and RXR form a dimer and induce basal transcription of genes. Upon the addition of either (2) an RXR ligand (9-*cis* retinoic acid) or (3) an LXR ligand (oxysterol), the heterodimer undergoes a further conformational change, resulting in a substantial increase in transactivation. (4) When both ligands are present, activation is synergistic. Modified from Peet et al. (1998).

responsible for *de novo* FA synthesis, FA activation, uptake, trafficking, and desaturation, and TAG synthesis (Bionaz and Loor, 2008b). As proteins, these enzymes are possibly regulated by the 3 transcription factors presented above. In addition, the biohydrogenation theory is based on the hypothesis of a direct inhibition of one or more steps in mammary milk fat synthesis. Thus, the lipogenic enzymes and their possible regulators are possibly influenced by *trans*-10, *cis*-12 CLA during MFD, therefore leading to reduced milk fat synthesis.

To investigate the role of SREBP-1 and PPARγ in milk fat synthesis, Bionaz and Loor (2008b) examined the expression of these two factors in dairy cows and reported that their expression was increased during lactation. Further, PPARγ activation by its agonist and coordinated up-regulation of lipogenic gene networks in bovine mammary epithelial cells indicated a role of PPARγ in regulating milk fat synthesis (Kadegowda et al., 2009). Similarly, the activation of LXR by its agonist increased *de novo* FA synthesis and the expression of SREBP-1 and FAS in bovine mammary epithelial cells, implying LXR as a regulator of milk fat synthesis (McFadden and Corl, 2010). The identification of LXRE in bovine SREBP-1 promoter further supported the hypothesis of LXR playing a role in milk fat synthesis regulation (Lengi and Corl, 2010).

Transcriptional regulation of lipogenic enzymes could also be one of the mechanisms for MFD. Therefore, numerous studies have been done to examine the expression of lipogenic genes and transcription factors in cell culture system or dairy cows with MFD. There are studies showing reduced expression of lipogenic enzymes, including ACC and FAS (*de novo* FA synthesis), and SCD1 (FA desaturation), in cows infused or fed with *trans*-10, *cis*-12 CLA (Baumgard et al., 2002; Peterson et al., 2003; Peterson et al., 2004). Similarly, enzymes critical for TAG synthesis (GPAT and AGPAT), and FA uptake and trafficking (LPL and FABP)

declined in cows receiving *trans*-10, *cis*-12 CLA abomasally or orally (Baumgard et al., 2002; Peterson et al., 2003). In addition, *trans*-10, *cis*-12 CLA inhibited the mRNA expression of IDH1, an enzyme important for reducing equivalent supply during milk fat synthesis, in bovine mammary epithelial cells (Liu et al., 2006). Taken together, these studies indicate that the lipogenic enzymes might be regulatory and control points for milk fat synthesis during MFD.

To determine the involvement of transcription factors in MFD, their expression was examined. The expression of SREBP-1 was decreased by *trans*-10, *cis*-12 CLA in bovine mammary epithelial cells and promoter analysis revealed SRE in the promoters of FAS and SCD1, indicating a role of SREBP-1 in regulating milk fat synthesis during MFD (Peterson et al., 2004). The reduction of SREBP-1 during MFD was also confirmed in cows fed with *trans*-10, *cis*-12 CLA (Harvatine and Bauman, 2006). In addition to SREBP-1, PPARγ and LXR are likely involved in regulating lipogenic enzymes during MFD as well, based on the coordinated up-regulation of lipogenic networks by their agonists in bovine mammary epithelial cells (Kadegowda et al., 2009; McFadden and Corl, 2010). Therefore, the reduced milk fat yield during MFD might be caused by decreased expression of lipogenic enzymes, which are regulated by the transcription factors.

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Chapter 3

Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1

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INTRODUCTION

Milk fat is the major energy component of milk, and regulation of its synthesis has been of interest to many research groups because milk fat is important for processing and flavor aspects of milk. Two sources of FA exist for milk fat synthesis: *de novo* FA synthesized by mammary epithelial cells and preformed FA from the digestive tract or body fat mobilization. In cows, about one-half of FA, including short-chain FA (4–8 carbons), medium-chain FA (10–14 carbons), and a portion of 16-carbon FA, come from *de novo* FA synthesis, with acetate and β-hydroxybutyrate as the precursors. The other half, including remaining 16-carbon FA and FA with more than 16 carbons, are preformed FA taken up from circulation by the mammary gland (Bauman and Griinari, 2003). Although much is known about the synthesis of milk fat, its regulatory pathways are still not well understood (Rudolph et al., 2006).

Milk fat synthesis is highly regulated by dietary factors, and MFD is one example of dietary regulation of milk lipid synthesis. Milk fat depression is a syndrome in which milk fat content is reduced up to 50% when cows are fed readily fermentable diets, such as high-concentrate/low-fiber diets, or diets supplemented with fish oil or plant oil. However, other

components in milk and milk yield are not affected (Peterson et al., 2003). Bauman and Griinari (2003) proposed that intermediates from alternative ruminal biohydrogenation pathways of polyunsaturated FA exhibit inhibitory effects on milk fat synthesis. Among the intermediates, *trans-10, cis-12* CLA is a potent mediator of the inhibition of milk fat synthesis during MFD (Baumgard et al., 2000, Peterson et al., 2002). Reduction in mRNA abundance of genes encoding enzymes involved in FA transport and uptake, *de novo* FA synthesis, desaturation of FA, and TAG synthesis has been associated with MFD (Baumgard et al., 2002).

Several transcription factors can regulate FA synthesis. One family of transcription factors designated SREBP, which belong to the basic helix-loop-helix leucine zipper family, are known to regulate FA synthesis (Shimano, 2001). Three isoforms of SREBP have been identified: SREBP-1a, SREBP-1c, and SREBP-2. Both SREBP- 1a and SREBP-1c are transcribed from the same gene with alternative splicing; SREBP-1c and SREBP-1a use different first exons and both regulate FA synthesis. Sterol regulatory element binding protein-2 is transcribed from a separate gene and regulates the cholesterol biosynthetic pathway. All SREBP are translated as precursor proteins residing in the ER membrane and are activated by proteolytic cleavage in the Golgi to release the mature SREBP protein that can translocate to the nucleus and bind to the promoter of target genes to induce their transcription (Eberle et al., 2004).

The transcription of many genes involved in milk fat synthesis is downregulated during MFD, and SREBP-1 has been implicated in mediating these effects. This is supported by studies showing that the nuclear SREBP-1 protein was reduced when a bovine mammary epithelial cell line was treated with *trans*-10, *cis*-12 CLA (Peterson et al., 2004). Transcriptional regulation of FA synthesis may be mediated by several different factors. The exact, independent role of each factor has not been previously established. Thus, our objective was to reveal the specific role of

SREBP-1 in regulating lipid synthesis in bovine mammary epithelial cells independent of CLA-induced reductions in lipid synthesis. In this study, knockdown of SREBP-1 in MAC-T cells was achieved using a double-stranded small interfering RNA (**siRNA**), with 21 to 23 nucleotides, designed specifically to reduce SREBP-1 expression.

MATERIALS AND METHODS

Cell Culture and Treatments

Experiments were performed using the MAC-T bovine mammary epithelial cell line. Basal culture medium consisted of high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 kU/mL of penicillin, 10 mg/mL of streptomycin, and 25 μg/mL of amphotericin. For hormonal medium, fetal bovine serum was removed from basal medium and hormones (0.1 μg/mL of insulin and 1.5 μg/mL of prolactin; Sigma Chemical Co., St. Louis, MO) were added. Cells were routinely cultured at 37°C with 5% CO_2 and basal medium was applied.

For experiments, cells were seeded in 6-well plates at a density of 2×10^4 cells/cm^2. After overnight incubation in basal medium, cells were switched to hormonal medium and siRNA (Dharmacon, Thermo Fisher Scientific Inc., Waltham, MA) were delivered to cells by Transfection Reagent #2 (Dharmacon) at 100 nM according to the manufacturer's protocol. Treatments included specific anti-SREBP-1 siRNA (SSI; ccacaacgccaucgagaaauu and gcaccgaggccaaguugaauu), siRNA with a random sequence as negative control (NEG), and transfection reagent alone as untransfected control (UNT). Treated cells were incubated for 48 h and then harvested for analysis. Experiments were repeated 3 to 4 times.

RNA Extraction and Real-Time PCR

Total RNA was extracted from cells using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions. After extraction, ribonucleic acid pellets were resuspended in RNase-free water and concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Then, RNA (1 μg per reaction) was reverse transcribed to complementary DNA using the Omniscript RT kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Oligo-dT (Eurofins MWG/Operon, Huntsville, AL) was used as the primer for reverse transcription. Real-time quantitative PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) in an Applied Biosystems 7300 real-time PCR machine (Applied Biosystems, Foster City, CA). Reactions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Each reaction was performed in duplicate wells. β-Actin was used as the endogenous control gene, and fold change was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with UNT as the calibrator. Gene-specific primers for the transcripts used in the study are shown in Table 3.1.

Protein Extraction and Immunoblotting

Cells were harvested in cold lysis buffer (50 mM Tris, pH 7.4, 0.5% Triton X-100, 0.3 *M* NaCl, 2 mM EDTA, pH 8.0, and protease inhibitor), followed by centrifugation at 16,100 × *g* for 15 min at 4°C. Supernatants were collected for protein concentration measurements. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). To ensure equal loading, samples were diluted to the same protein concentrations with Laemmli sample buffer (Sigma Chemical Co.) and heated at 95°C for 10 min. Proteins were separated by electrophoresis using polyacrylamide gels [7.5% gel for ACC and 12% gel for SREBP-1 and

SCD1; Cambrex Corporation, East Rutherford, NJ] and transferred to a polyvinyl difluoride membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were then blocked in blocking buffer (0.05 *M* Tris, pH 7.4, 0.2 *M* NaCl, 0.1% Tween, and 5% nonfat dry milk) on a rocker for 1 h at room temperature. Membranes were probed with primary anti-SREBP-1 antibody (SC-13551, Santa Cruz Biotechnology, Santa Cruz, CA), primary anti-ACC antibody (Cell Signaling Technology, Beverly, MA), or primary anti-SCD1 antibody (custom rabbit anti- bovine SCD1, Pacific Immunology, Ramona, CA) at 1:1,000 in blocking buffer at 4°C overnight. Membranes were washed in washing buffer (0.05 *M* Tris, pH 7.4, 0.2 *M* NaCl, and 0.1% Tween) twice for 10 min. Following washing, membranes were incubated with horseradish peroxidase-conjugated goat, anti-mouse or anti-rabbit secondary antibody (Santa Cruz Biotechnology) at 1:1,000 in blocking buffer for 1 h at room temperature. Membranes were washed 3 times, for 15 min each, and proteins were detected using ECL-Plus chemiluminescence substrate (Amersham Biosciences, Pittsburgh, PA) according to the manufacturer's instructions. Chemiluminescence was measured using a Chemidoc XRS digital imaging system and densitometry was per- formed using Quantity One software (Bio-Rad).

Fatty Acid Synthesis Assay

De novo FA synthesis was determined by quantifying the incorporation of [1-^{14}C]-acetate (MP Biomedicals, Solon, OH) into lipids. Methods were adapted from Peterson et al. (2004) with modifications. After cells were transfected with siRNA for 44 h, medium was removed and replaced with fresh medium containing 0.3 mM acetate and [1-^{14}C]-labeled acetic acid to a final concentration of 1 μCi/well and incubated for 4 h. After incubation, isotope-containing medium was removed and cells were washed with 1× PBS. Cells then were lysed with SDS buffer (0.1% in PBS) and lipids from the lysates were extracted with hexane-isopropanol (3:2). The organic

phase was transferred to scintillation vials. Scintillation cocktail (15 mL per vial; Scintisafe 30% Cocktail, Fisher Scientific) was added to scintillation vials. Radioactivity was measured using a LS 6000LL Beckmann scintillation counter (Beckmann Coulter Inc., Brea, CA). Activity was calculated and expressed as nanomoles of acetate incorporated per 4 h.

Fatty Acid Uptake Assay

Fatty acid uptake was measured by quantifying the incorporation of [1-^{14}C]-oleate (MP Biomedicals) into lipids. Uptake of [1-^{14}C]-labeled oleate was measured as described for [1-^{14}C]-acetate incorporation. Oleate (Matreya LLC, Pleasant Gap, PA) was bound to BSA (Sigma Chemical Co.) using the method described by Ip and coworkers (1999) with modifications. Briefly, pure oleate was weighed and mixed with 0.05 *M* NaOH. The resulting sodium salt was complexed with BSA solution at a 3:1 molar ratio to a final concentration of 100 μM oleate and 1 μCi/well [1-^{14}C]-labeled oleate.

Statistical Analysis

All data were analyzed using the Mixed procedure (SAS 9.2; SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment and the random effect of replicate. Treatment means were compared using orthogonal contrasts [UNT vs. NEG and (UNT + NEG) vs. SSI]. Differences were considered significant when $P < 0.05$.

RESULTS

The mRNA abundance of SREBP-1 was decreased more than 90% when MAC-T cells were transfected with SREBP-1–specific siRNA (Figure 3.1 A). Both precursor and mature forms of SREBP-1 protein were undetectable from SREBP-1 siRNA-treated cells, whereas abundant protein was present in control cells (Figure 3.1 B), indicating the effectiveness of

siRNA in reducing SREBP-1 mRNA abundance. The mRNA expression of spot 14 (S14) declined dramatically with SREBP-1 siRNA treatment, and this was also observed in the negative control (Figure 3.1 C). Cells with SREBP-1 knockdown had significantly less FA synthesis and uptake compared with controls, as shown by acetate (Figure 3.1 D) and oleate (Figure 3.1 E) incorporation data.

Because acetate is the substrate for the *de novo* lipogenic pathway and we observed a reduction in acetate incorporation, we examined the mRNA abundance of genes encoding enzymes that play key roles in the *de novo* lipogenic pathway. Levels of mRNA of ACSS2, ACC, FAS, and IDH1 were reduced by 60, 40, 65, and 50%, respectively, by SREBP-1 siRNA treatment compared with controls (Figure 3.2 B-E). Protein expression of ACC was also reduced by approximately 50% in cells treated with SREBP-1 siRNA (Figure 3.3 A). However, mRNA expression of ACSS1 was not changed when SREBP-1 was knocked down (Figure 3.2 A).

To evaluate the effect of SREBP-1 on the expression of enzymes involved in long-chain FA activation, transport, and desaturation, we also analyzed the mRNA levels of ACSL1, FABP3, and SCD1. Among these transcripts, we observed no difference between SREBP-1 siRNA treatment and controls for ACSL1 (Figure 3.4 A). However, mRNA levels of FABP3 and SCD1 were decreased by 76 and 60%, respectively, in cells transfected with siRNA specific for SREBP-1 (Figure 3.4 B and C). Additionally, SCD1 protein level was decreased more than 90% when SREBP-1 was inhibited by siRNA compared with controls (Figure 3.3 B).

After synthesis and activation, FA are esterified to glycerol-3-phosphate to produce TAG. Enzymes responsible for TAG esterification, including mitochondrial glycerol-3-phosphate acyltransferase (**GPAM**), AGPAT6, LPIN1, and DGAT1, were examined in the current study.

Levels of mRNA for GPAM and LPIN1 were decreased by about 20 and 90%, respectively, when cells were transfected with specific anti-SREBP-1 siRNA (Figure 3.5 A and C). However, the expression of AGPAT6 and DGAT1 were increased in SREBP-1 siRNA–treated cells compared with the control cells (Figure 3.5 B and D).

Milk fat is secreted as a globule surrounded by the milk fat globule membrane and proteins such as perilipin 2 (**PLIN2**) and butyrophilin subfamily 1 member A1 (**BTN1A1**). To investigate the role of SREBP-1 in transcription of milk fat globule protein genes, we measured the mRNA expression of PLIN2 and BTN1A1 with SREBP-1 knockdown. The abundance of mRNA for PLIN2 was upregulated in cells transfected with SREBP-1 siRNA (Figure 3.6 A). However, the mRNA expression of BTN1A1 was not changed, but showed a trend for downregulation with SREBP-1 siRNA treatment ($P = 0.06$; Figure 3.6 B).

DISCUSSION

Different SREBP isoforms have different roles in regulating lipid synthesis, with SREBP-1a being responsible for regulating both FA and cholesterol synthesis, whereas SREBP-1c and SREBP-2 contribute to the regulation of FA synthesis and cholesterol synthesis, respectively (Horton, 2002). As a regulator of lipid synthesis, SREBP-1 has been studied extensively in different species (Brown and Goldstein, 1997). In bovine mammary gland, SREBP-1c is one of the mechanisms regulating milk fat synthesis (Bauman et al., 2008).

Although many studies have examined the responses of SREBP-1 and lipogenic genes to MFD, the specific role of SREBP-1 in regulating milk fat synthesis has not been directly evaluated. *Trans-10, cis-12* CLA has been identified as a CLA isomer that causes MFD (Baumgard et al., 2000). Peterson et al. (2004) observed a reduction in mature SREBP-1 protein

and mRNA expression of ACC, FAS, and SCD1 in MAC-T cells treated with *trans-10,cis-12* CLA. Harvatine and Bauman (2006) also demonstrated a significant reduction of mammary mRNA abundance of SREBP-1, FAS, and LPL in lactating dairy cows administered *trans-10, cis-12* CLA or fed a low-forage and high-oil diet. In another abomasal infusion study, *trans-10, cis-12* CLA not only resulted in reduced milk fat content and yield, but also led to decreased mRNA abundance of the genes encoding LPL, FABP3, ACC, FAS, SCD1, GPAT, and AGPAT (Baumgard et al., 2002). These enzymes are involved in FA transport and uptake, *de novo* fatty acid synthesis, desaturation of FA and TAG synthesis. Thus, it is speculated that SREBP-1 plays a role in regulating milk fat synthesis during MFD, but the relationship between SREBP-1 and lipogenic enzymes independent of potential regulation by other factors during MFD has not been fully elucidated.

In this study, RNA interference was used to knock down SREBP-1 in MAC-T cells cultured in medium containing insulin and prolactin to investigate the specific role of SREBP-1 in affecting the expression of genes encoding key enzymes of milk fat synthesis. Our SREBP-1–specific siRNA targeted a region of the transcript identical between SREBP-1a and SREBP-1c and did not distinguish the 2 isoforms. Both mRNA and protein levels of SREBP-1 decreased dramatically, indicating high efficiency of the siRNA in interfering with SREBP-1 expression. Spot 14 is a gene encoding a nuclear protein that is associated with the regulation of FA synthesis in lipogenic tissues, and it has been shown to be engaged in mammary regulation of milk fat synthesis (Harvatine and Bauman, 2006). In our study, S14 was dramatically reduced by SREBP-1 siRNA, indicating that S14 might be regulated by SREBP-1 in MAC- T cells. However, it was also decreased in the negative control, which might be due to cell stress caused by transfection or similarity of random siRNA sequence and the S14 sequence. When SREBP-1

expression was reduced, FA synthesis and FA uptake were reduced as indicated by acetate and oleate incorporation data. Although both acetate and oleate incorporation were reduced, the magnitude of the reduction in acetate incorporation was greater than that of oleate incorporation, suggesting that *de novo* FA synthesis was affected to a greater extent during SREBP-1 knockdown. This is consistent with the milk FA profile in cows with MFD. Chouinard et al. (1999a) demonstrated that the effects of MFD-inducing CLA were most pronounced on *de novo* FA synthesis and the desaturation process. Similarly, SCD1 activity and *de novo* synthesized FA were extensively reduced when cows were infused with a high dose of *trans-10, cis-12* CLA (Baumgard et al., 2001).

To test whether *de novo* lipogenic enzymes are regulated by SREBP-1, we measured the expression of genes encoding lipogenic enzymes involved in the *de novo* FA synthesis pathway. As the precursor for *de novo* FA synthesis, acetate needs to be activated by acyl-CoA synthetase enzymes to produce acetyl-CoA. Between the 2 isoforms, ACSS2 is more abundant than ACSS1 in the mammary gland during lactation (Bionaz and Loor, 2008b). Both ACSS1 and ACSS2 have high affinity for acetate, with ACSS2 showing greater affinity. Bovine ACSS1 also activates about 4-fold more ^{14}C-acetate toward CO_2 than lipid, indicating that the acetyl-CoA produced by ACSS1 is mainly for the oxidative pathway (Fujino, 2001). Therefore, ACSS2 is the isoform responsible for acetate activation during *de novo* FA synthesis. In our study, ACSS2, but not ACSS1, was downregulated by SREBP-1 inhibition, indicating that ACSS2 appears to be part of the SREBP-1–regulated lipid synthetic pathway.

After activation, acetyl-CoA is catalyzed by ACC to synthesize malonyl-CoA. Acetyl-CoA carboxylase is the rate-limiting step of *de novo* FA synthesis. During lactation, the targeting of FA precursors to the mammary gland is primarily driven through suppression of the activity of

ACC in adipose tissue and induction of ACC in mammary gland, which is in part through corresponding changes in ACC mRNA levels in the 2 tissues, to satisfy the increased metabolic demand created by the requirement to milk fat synthesis and secretion (Clegg et al., 2001). Fatty acid synthase is another key enzyme of *de novo* FA synthesis and is responsible for adding carbons to the FA chain to produce short-chain and medium-chain FA. In the current study, expression of both ACC and FAS was reduced with SREBP-1 suppression, indicating that transcription of ACC and FAS is regulated by SREBP-1. The reduction in ACC and FAS has also been observed in some MFD studies (Peterson et al., 2003). In another study with MAC-T cells, reduction in mature SREBP-1 protein with *trans*-10*, cis*-12 CLA corresponded to reductions in mRNA expression of ACC and FAS (Peterson et al., 2004). Taken together, ACC and FAS are both involved in the SREBP-1-regulated lipogenic pathway in MAC- T cells and the reduction of these 2 enzymes during MFD is likely due to SREBP-1 inhibition.

Besides the enzymes mentioned above, IDH1 plays a role in FA biosynthesis by providing NADPH. Liu et al. (2006) found that IDH1 expression was reduced by *trans*-10*, cis*-12 CLA in the BME-UV bovine mammary epithelial cell line and proposed that the inhibitory effects of *trans*-10*, cis*-12 CLA on milk fat synthesis in mammary epithelial cells might derive, at least in part, from repression of IDH1 expression and reduced NADPH availability. In the current study, we also observed a significant reduction in IDH1 expression when SREBP-1 was inhibited by siRNA, indicating that IDH1 is one of the targets for SREBP-1, and decreased IDH1 during MFD might be regulated by reduced SREBP-1 level. High-glucose medium was used during transfection and this might decrease IDH1 activity. We did not measure IDH1 activity in the current study, but its mRNA level was indeed reduced by SREBP-1 knockdown.

Preformed long-chain FA from the diet and adipose lipolysis are also taken up by the

mammary gland to synthesize milk fat. The activation of long-chain FA is catalyzed by ACSL enzymes. Six isoforms of ACSL have been identified, and ACSL1 has been shown to be the most abundant isoform in the lactating bovine mammary gland (Bionaz and Loor, 2008a). Upregulation of ACSL1 at the onset of lactation was also observed by Finucane et al. (2008). In the current study, ACSL1 was not affected by SREBP-1 knockdown, suggesting that SREBP-1 is not involved in the transcriptional regulation of long-chain FA activation by ACSL1. Similarly, when SREBP-1 expression was significantly decreased in bovine kidney cells treated with *trans*-10, *cis*-12 CLA, the expression of ACSL1 was not changed (Bionaz et al., 2012). Thus, ACSL1 seems not to be a target of SREBP-1. In fact, it has been demonstrated that ACSL1 is a peroxisome proliferator-activated receptor α target because its expression was upregulated by a peroxisome proliferator-activated receptor α agonist (Bionaz et al., 2012).

The transport of long-chain FA within the cell may be mediated by FABP, and it has been shown that FABP3 is the most abundant isoform in lactating bovine mammary gland (Bionaz and Loor, 2008a). Before addition to the glycerol backbone, some saturated long-chain FA, especially stearic acid, are modified by SCD1 to produce unsaturated FA. Thus, we measured the expression of FABP3 and SCD1 and found that both declined with SREBP-1 inhibition, indicating that transport and desaturation of long-chain FA are regulated transcriptionally by SREBP-1. Kadegowda et al. (2009) observed a dramatic decline in FABP3 in MAC-T cells treated with *trans*-10, *cis*-12 CLA. Evidence also exists for a significant reduction in SCD1 mRNA abundance from mammary tissue during abomasal infusion of *trans*-10, *cis*-12 CLA (Baumgard et al., 2002). Combining these data, FABP3 and SCD1 are involved in the SREBP-1-regulated pathway during milk fat synthesis.

During milk fat synthesis, FA from the *de novo* pathway or circulation are esterified to

glycerol-3-phosphate to produce TAG. During this process, enzymes for adding FA to the 3 positions of glycerol backbone and for removing the phosphate group play key roles in controlling synthesis rates. These enzymes include GPAM (addition of FA to glycerol-3-phosphate), AGPAT6 (addition of FA to sn-2 position of acylglycerol- 3-phosphate), LPIN1 (phosphatidic acid phosphohy- drolase activity), and DGAT1 (addition of FA to sn-3 position of diacylglycerol) (Bionaz and Loor, 2008a, b). After treatment with SREBP-1 siRNA, both GPAM and LPIN1 were decreased by SREBP-1 siRNA treatment, but the mRNA levels of AGPAT6 and DGAT1 were increased when SREBP-1 expression was reduced. The reduction in GPAM with SREBP-1 siRNA treatment in this study indicates that GPAM is controlled by SREBP-1 to affect milk fat synthesis. Two LPIN1variants (a and b) have been described in mice (Peterfy et al., 2005); however, no bovine sequences distinguish between the 2 isoforms. The present data should be considered as the combination of the 2 variants. It has been demonstrated that LPIN1 was upregulated during lactation and downregulated in MAC-T cells treated with *trans*-10*, cis*-12 CLA (Bionaz and Loor, 2008a). Thus, LPIN1 plays a role in milk TAG synthesis, and the reduction during *trans*-10*,* cis-*12* CLA treatment might result from decreased expression of SREBP-1. The increase in AGPAT6 was not expected and might be caused by compensatory effects of other transcription factors. Evidence exists that AGPAT6 has GPAT activity but no AGPAT activity in human kidney cells and mice, and it has been renamed GPAT4, suggesting that other AGPAT isoforms may play important roles in the second step of triglyceride synthesis (Chen et al., 2008, Nagle et al., 2008). Regardless of the specific role of AGPAT6, it is not regulated by SREBP-1 in bovine mammary epithelial cells. Because DGAT2 is thought to be adipocyte-specific and almost undetectable in the mammary gland, DGAT1 is more likely responsible for adding FA to the sn-3 position of diacylglycerol during milk fat synthesis

(Bionaz and Loor, 2008b). The increase in DGAT1 in this study indicated that DGAT1 might not be regulated by SREBP-1. In fact, it has been shown that the expression of DGAT1 was not changed in MAC-T cells treated with *trans*-10*, cis*-12 CLA; however, its activity was significantly decreased (Sørensen et al., 2008). Instead, *trans*-10*, cis*-12 CLA may affect DGAT1 activity directly or through a posttranslational mechanism. Although these triglyceride esterification enzymes responded differently to SREBP-1 knockdown, the reduction in GPAM and LPIN1 expression might be enough to reduce TAG synthesis without reductions in AGPAT6 and DGAT1 because both of them are key enzymes for this step.

Because milk fat is secreted as milk fat globules enveloped by the milk fat globule membrane, we analyzed the expression of PLIN2 and BTN1A1, 2 milk fat droplet proteins, to determine the role of SREBP-1 in milk fat droplet formation (Nielsen et al., 1999). The sequence of bovine PLIN2 is identical to the sequence of bovine adipophilin in mammary gland. Adipophilin is associated with milk lipid globule membranes and its levels correlate with lipid droplet deposition (Heid et al., 1998). We observed an increase in PLIN2 and no change in BTN1A1 in cells treated with SREBP-1 siRNA, indicating that the regulatory role of SREBP-1 in milk lipid synthesis might be limited to FA synthesis, activation, transport, desaturation, and TAG esterification, but there is no SREBP-1 effect on transcription of genes involved in milk fat droplet formation or secretion. Another possibility is that fat droplets were not formed because MAC-T cells did not fully differentiate in culture. The fat droplet synthetic pathway might not have been activated and the genes were unable to respond to SREBP-1 knockdown.

Besides S14, some other measurements were also significantly affected by the negative control siRNA compared with the untransfected control. The changes may have resulted from cell stress caused by transfection or other nonspecific siRNA effects. The contrast between

SREBP-1 siRNA treatment and controls ruled out possible nonspecific effects caused by transfection reagent and siRNA.

In our study, we focused on only one transcription factor in regulating the transcription of genes involved in milk fat synthesis. However, other factors regulate transcription, translation, and activity of lipogenic enzymes. We did not measure levels of other transcription factors, such as LXR and PPARγ, so the effect of these factors cannot be ruled out. When SREBP-1 is knocked down, LXR, PPARγ, and other factors might be responsible for maintaining the expression of some targets, so that no change or an increase in transcripts occurs. Because the ultimate, active form of enzymes is protein, processing during translation and activation cannot be ignored, and regulation of lipogenic enzymes may occur at both the transcriptional and translational levels. Although many studies have used knockout mice to study the role of SREBP-1 in lipid synthesis, it is very difficult to make knockout cows. Our in vitro experiments take advantage of RNA interference to knock down SREBP-1 and provide insights into how SREBP-1 regulates mammary epithelial cell lipid synthesis. However, milk fat synthesis is a more complicated process in vivo and can be affected by surrounding tissues and energy status of the cow.

In conclusion, SREBP-1 regulates lipid synthesis in bovine mammary epithelial cells by controlling the transcription of genes encoding enzymes involved in *de novo* FA synthesis, FA desaturation, long-chain FA uptake, and TAG esterification. However, transcription of PLIN2 and BTN1A1 is not regulated by SREBP-1.

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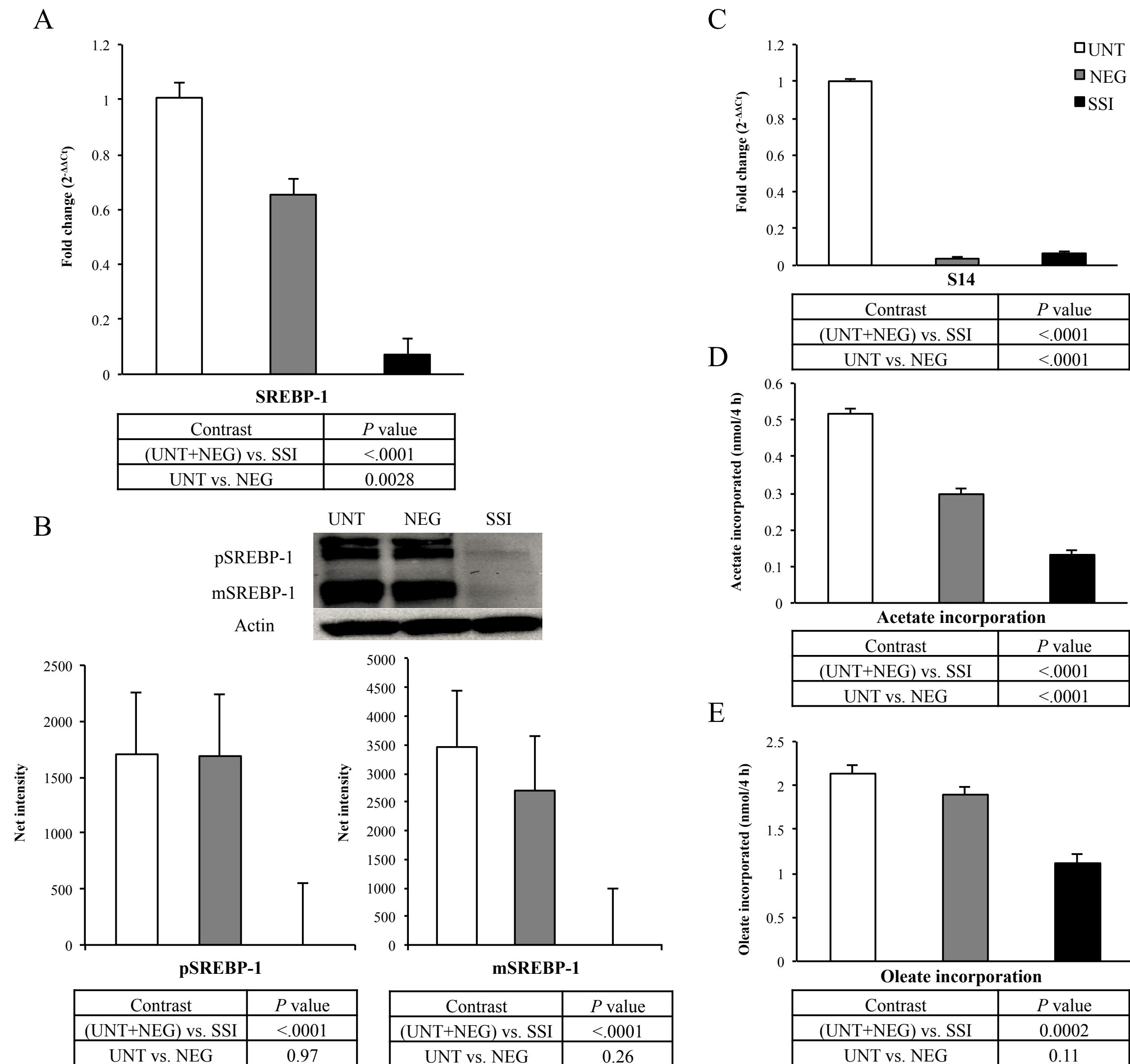
TABLES

Table 3.1 Primer sequences for transcripts used in real-time quantitative PCR

Transcript	Accession number		Primers (5'-3')
Actin	AY141970	Forward	ctcttccagccttccttcct
		Reverse	gggcagtgatctctttctgc
SREBP-1	NM_001113302	Forward	atgccatcgagaaacgctac
		Reverse	gtccgcagactcaggttctc
FABP3	NM_174313.2	Forward	aagcctaccacaatcatcgaag
		Reverse	ttcaagctgggagtcgagttc
SCD1	AY241933	Forward	ccctttccttgagctgtctg
		Reverse	atgctgactctctcccctga
ACC	NM_174224	Forward	gggtgaaagactgggttgaa
		Reverse	gacagagcacggatgtgatg
FAS	NM_001012669	Forward	ctgcaactcaacgggaactt
		Reverse	aggctggtcatgttctccag
GPAM	AY515690	Forward	attgacccttggcacgatag
		Reverse	aacagcaccttcccacaaag
DGAT1	AY065621	Forward	gacacagacaaggacggaga
		Reverse	cagcatcaccacacaccaa
AGPAT6	NM_001083669	Forward	aagcaagttgcccatcctca
		Reverse	agctttaacctcggtgtcaaa
ACSS1	BC114698	Forward	gatgatgtgggtgatgtgga
		Reverse	ccagaccgagtttttggaag
ACSS2	BC134532	Forward	ggactgaaacagggaagcaa
		Reverse	cgcacaagagaagcaacaaa
ACSL1	NM_001076085	Forward	tggcccatatgtttgagaga
		Reverse	gggccttgagatcatccata
IDH1	NM_181012	Forward	cgatgagaagagagtggagga
		Reverse	caagccggggtatattttg
BTN1A1	NM_174508	Forward	agcccctgtttcttcctgtt
		Reverse	tgccgacctaactccatctt
S14	NM_001040533	Forward	gaggaggagagcgtgttgg
		Reverse	tcagaggaaggtagggagga
LPIN1	XM_002707716	Forward	gaggggaagaaacaccacaa
		Reverse	gtagctgacgctggacaaca
PLIN2	NM_173980	Forward	tggtctcctcggcttacatc
		Reverse	tcttttgccccagtcatagc

FIGURES

Figure 3.1 The expression of sterol regulatory element binding protein (SREBP)-1 mRNA, SREBP-1 proteins, S14 mRNA, acetate incorporation, and oleate incorporation in cells treated with SREBP-1 specific small interfering (si)RNA



A – SREBP-1

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.0028

B – pSREBP-1

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.97

B – mSREBP-1

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.26

C – S14

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	<.0001

D – Acetate incorporation

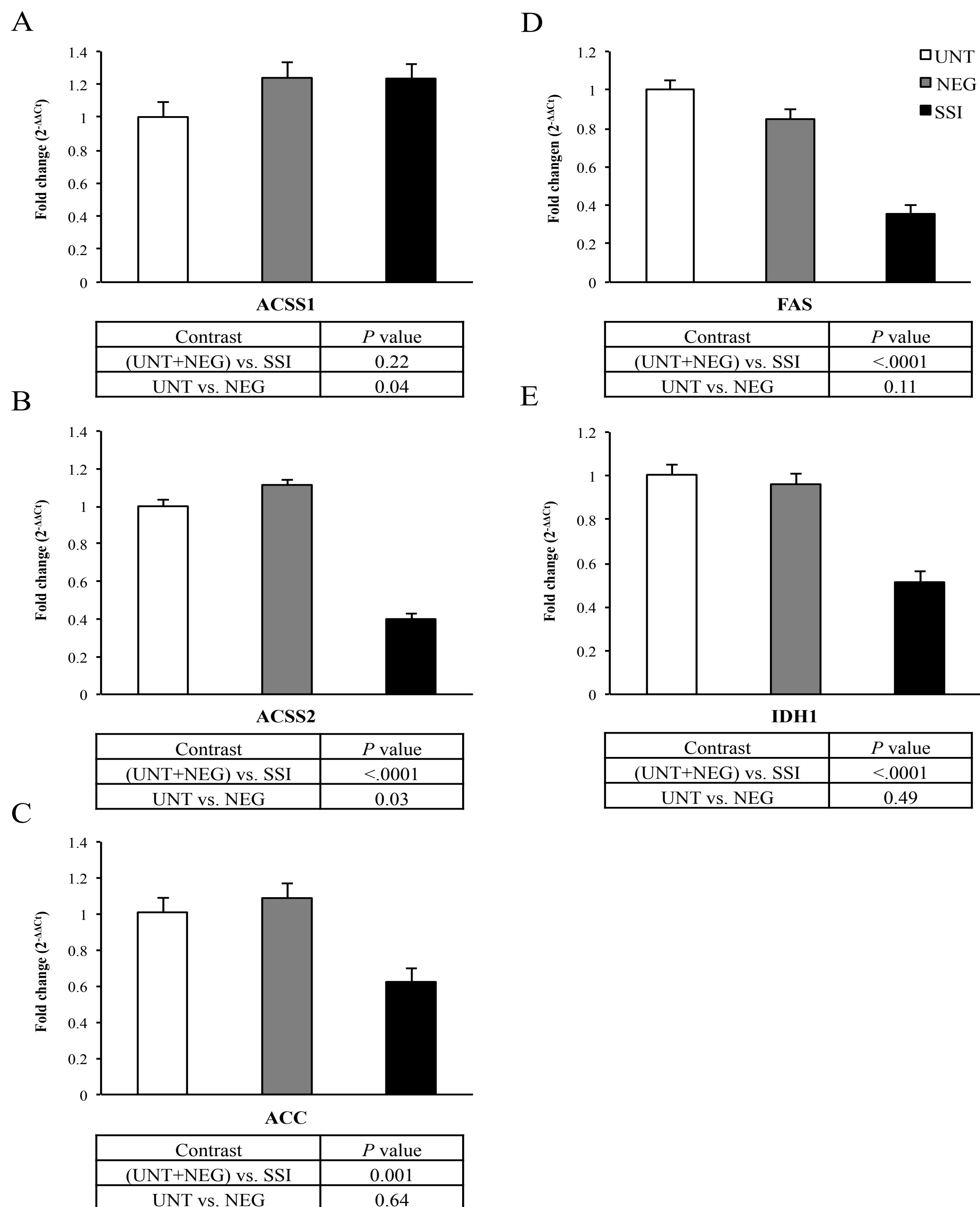
Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	<.0001

E – Oleate incorporation

Contrast	*P* value
(UNT+NEG) vs. SSI	0.0002
UNT vs. NEG	0.11

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a nontargeting siRNA sequence with transfection reagent (NEG), or an SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Experiments were repeated 4 times. Fold change of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Protein bands were visualized and quantified using Quantity One software (Bio-Rad, Hercules, CA). Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Figure 3.2 The mRNA expression of *de novo* lipogenic enzymes in cells treated with sterol regulatory element binding protein (SREBP)-1 small interfering (si)RNA



A — ACSS1

Contrast	*P* value
(UNT+NEG) vs. SSI	0.22
UNT vs. NEG	0.04

B — ACSS2

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.03

C — ACC

Contrast	*P* value
(UNT+NEG) vs. SSI	0.001
UNT vs. NEG	0.64

D — FAS

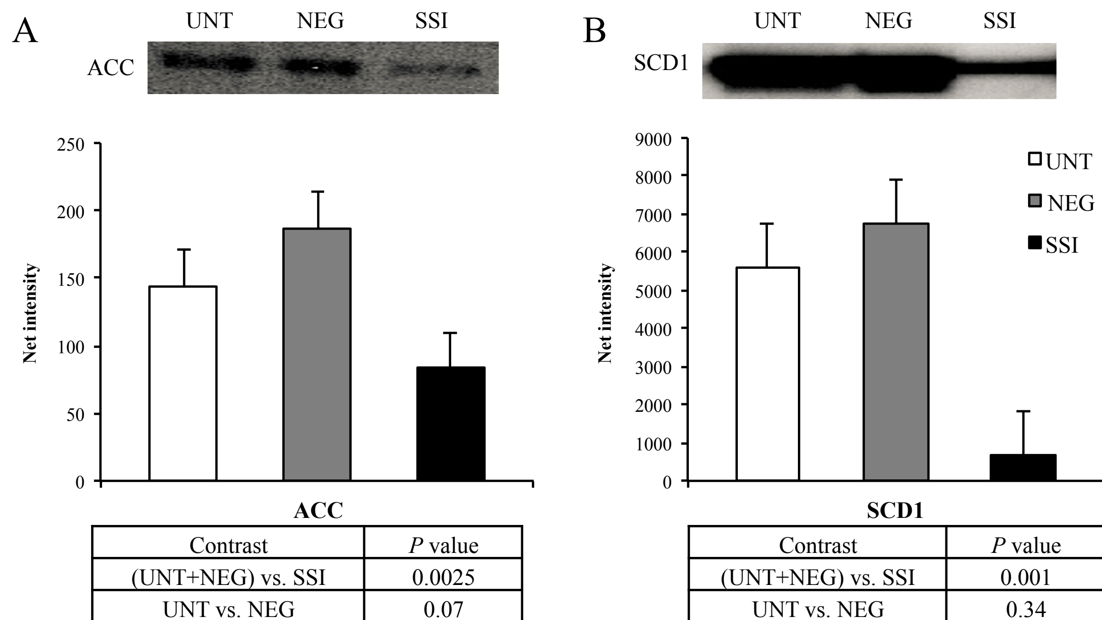
Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.11

E — IDH1

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.49

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a nontargeting siRNA sequence with transfection reagent (NEG), or an anti-SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Fold change of mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Figure 3.3 The protein levels of ACC and SCD1 in cells transfected with sterol regulatory element binding protein (SREBP)-1 small interfering (si)RNA

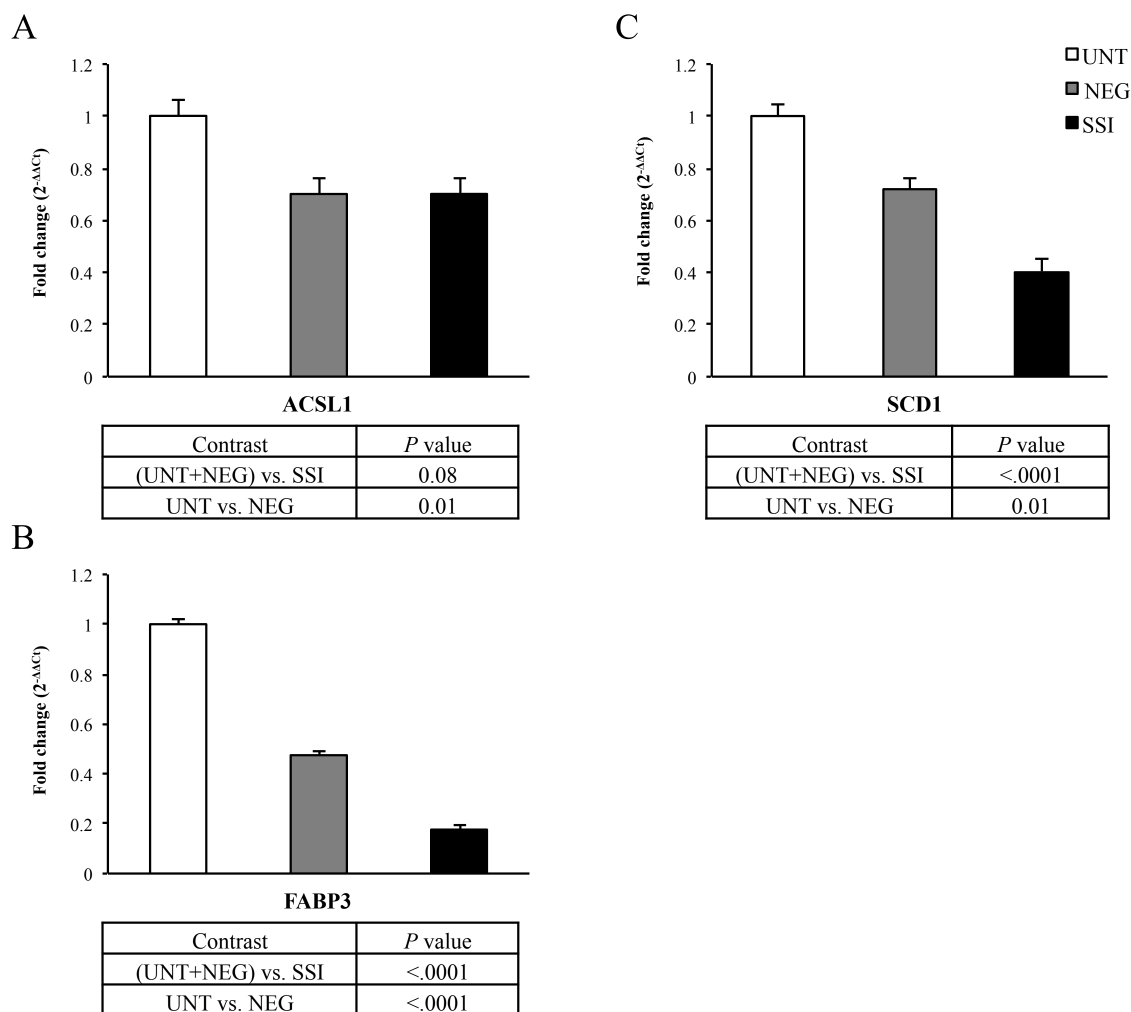


Contrast	*P* value
(UNT+NEG) vs. SSI	0.0025
UNT vs. NEG	0.07

Contrast	*P* value
(UNT+NEG) vs. SSI	0.001
UNT vs. NEG	0.34

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a nontargeting siRNA sequence with transfection reagent (NEG), or an SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Proteins were separated using electrophoresis. Protein bands were visualized and densitometry was quantified using Quantity One software (Bio-Rad, Hercules, CA). Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Figure 3.4 The mRNA abundance of ACSL1, FABP3, and SCD1 in cells transfected with sterol regulatory element binding protein (SREBP)-1 small interfering (si)RNA



A — ACSL1

Contrast	*P* value
(UNT+NEG) vs. SSI	0.08
UNT vs. NEG	0.01

C — SCD1

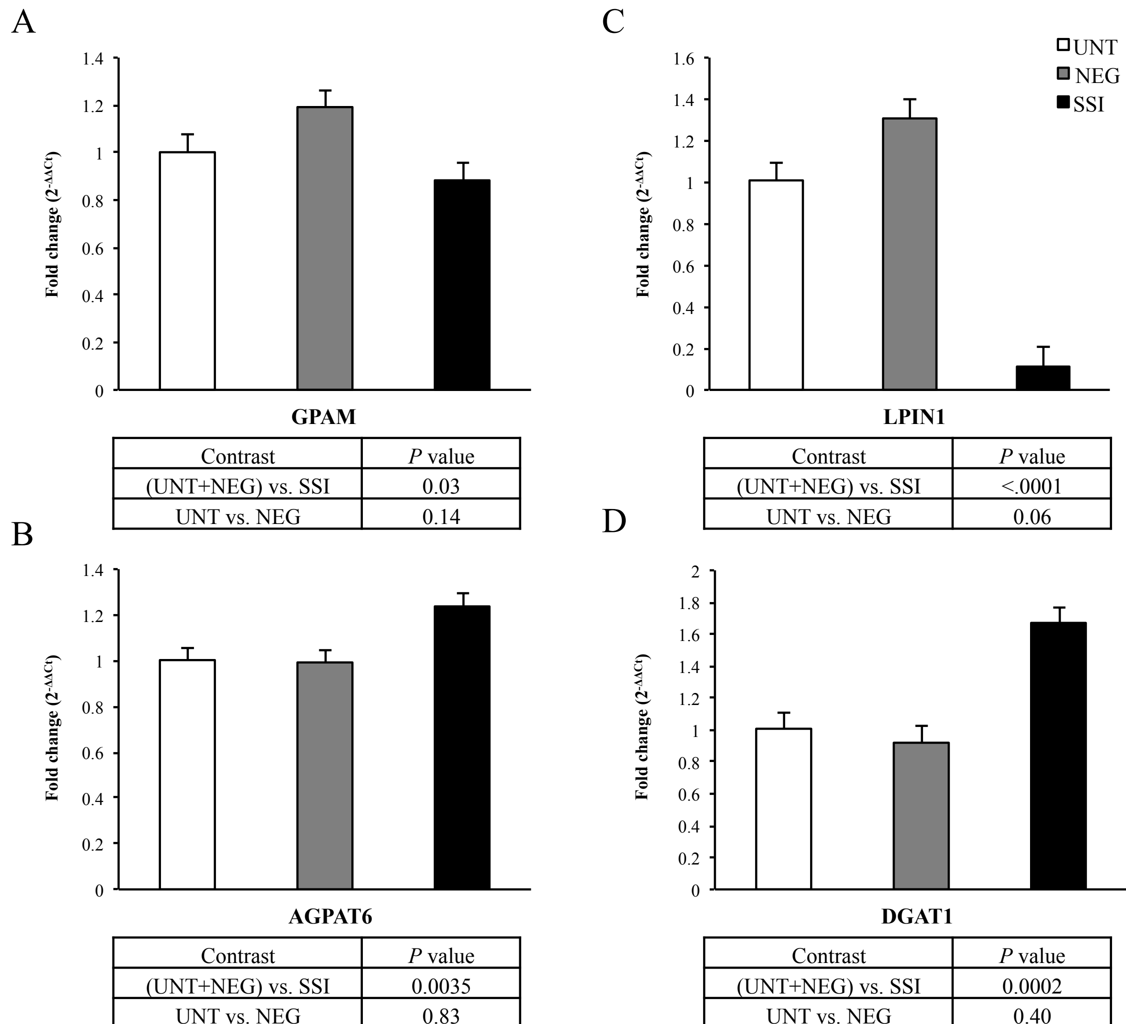
Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.01

B — FABP3

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	<.0001

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a nontargeting siRNA sequence with transfection reagent (NEG), or an SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Real-time PCR was performed using corresponding primers. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Figure 3.5 The mRNA abundance of genes encoding enzymes involved in triglyceride esterification in cells with sterol regulatory element binding protein (SREBP)-1 knockdown



A — GPAM

Contrast	*P* value
(UNT+NEG) vs. SSI	0.03
UNT vs. NEG	0.14

C — LPIN1

Contrast	*P* value
(UNT+NEG) vs. SSI	<.0001
UNT vs. NEG	0.06

B — AGPAT6

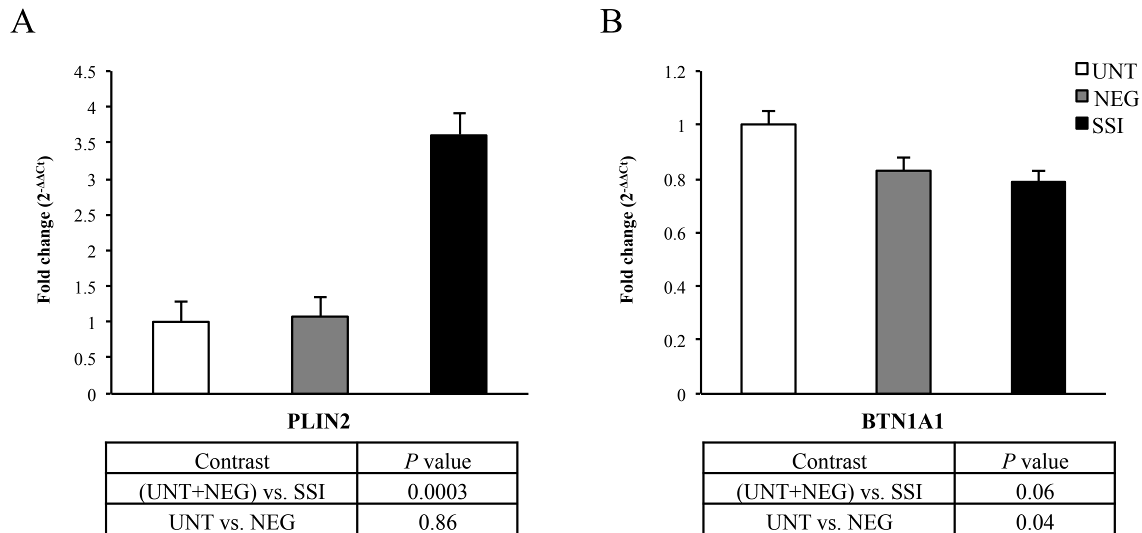
Contrast	*P* value
(UNT+NEG) vs. SSI	0.0035
UNT vs. NEG	0.83

D — DGAT1

Contrast	*P* value
(UNT+NEG) vs. SSI	0.0002
UNT vs. NEG	0.40

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a nontargeting small interfering (si)RNA sequence with transfection reagent (NEG), or an SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Real-time PCR was performed. Fold change of mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Figure 3.6 The mRNA expression of genes related to milk fat droplet formation and secretion in cells transfected with sterol regulatory element binding protein (SREBP)-1 small interfering (si)RNA



A — PLIN2

Contrast	*P* value
(UNT+NEG) vs. SSI	0.0003
UNT vs. NEG	0.86

B — BTN1A1

Contrast	*P* value
(UNT+NEG) vs. SSI	0.06
UNT vs. NEG	0.04

Bovine mammary epithelial cells (MAC-T cell line) were treated with transfection reagent only (UNT), a non-targeting siRNA sequence with transfection reagent (NEG), or an SREBP-1 specific siRNA with transfection reagent (SSI) at 100 nM for 48 h. Real-time PCR was performed. Fold change of mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. Means (± SEM) were compared using contrasts; difference was considered significant when $P < 0.05$.

Chapter 4

Effects of insulin, prolactin and leptin and *trans*-10, *cis*-12 CLA on lipogenic transcription factor activation in bovine mammary epithelial cells

INTRODUCTION

Milk fat is an important component of cow's milk and is reduced by feeding certain diets, including those providing excessive amounts of readily digestible carbohydrates and those supplemented with polyunsaturated FA (Bauman and Griinari, 2003). This marked reduction in milk fat production is referred to as MFD. Current hypotheses state that MFD is caused by FA intermediates produced by altered rumen biohydrogenation of unsaturated FA (Bauman and Griinari, 2001). *Trans*-10, *cis*-12 CLA has since been implicated as a major FA intermediate responsible for MFD (Baumgard et al., 2000). Baumgard et al. (2002) demonstrated that abomasal infusion of *trans*-10, *cis*-12 CLA inhibited milk fat synthesis by decreasing the expression of lipogenic genes, including FAS and SCD1. Additionally, the expression of SREBP-1, a transcription factor involved in lipogenesis, was reduced in *trans*-10, *cis*-12 CLA treated bovine mammary epithelial cells and the mammary gland of MFD cows, and promoter analysis reveals binding sites for SREBP-1 in bovine FAS and SCD1 promoters (Harvatine and Bauman, 2006; Peterson et al., 2004). In addition, lipogenic gene expression was reduced when SREBP-1 was inhibited in bovine mammary epithelial cells, indicating a regulatory role of SREBP-1 in lipid synthesis in theses cells (Ma and Corl, 2012). Therefore, reduced SREBP-1 expression and activation are likely part of the mechanism of MFD.

Other transcription factors are involved in regulating lipogenesis in various tissues as well, such as PPARγ and LXR. Expression of lipogenic genes in bovine mammary epithelial cells was induced by the synthetic PPARγ agonist, rosiglitazone, indicating that PPARγ might play a regulatory role in milk fat synthesis (Kadegowda et al., 2009). In a mouse study, a diet supplemented with *trans*-10, *cis*-12 CLA inhibited the gene expression of PPARγ and its downstream target, LPL in white adipose tissue, suggesting the involvement of *trans*-10, *cis*-12 CLA in reducing lipogenesis and PPARγ expression (Kang et al., 2003). Liver X receptor regulates SREBP-1 expression in mouse liver, adipose tissue, and intestine (Repa et al., 2000). Liver X receptor activation also up-regulates the expression and activation of SREBP-1 in bovine mammary epithelial cells, revealing potential regulation of milk fat synthesis in dairy cows by LXR (McFadden and Corl, 2010). These transcription factors are potential regulators for milk fat synthesis and might be the control points of FA synthetic pathways during MFD.

Lipogenesis of cultured mammary tissue or cells has been previously shown to be regulated by hormones and this regulation might be mediated at the transcriptional level. Prolactin alone or in combination with insulin and corticosterone stimulated the rate of medium- and long-chain FA synthesis in rabbit mammary explants (Forsyth et al., 1972). Additionally, leptin enhanced FA synthesis in the cultured bovine mammary tissue, but only in the presence of prolactin (Feuermann et al., 2004). Insulin is a well-known hormone that stimulates lipogenesis in rat liver and adipose tissue (Assimacopoulos-Jeannet et al., 1995). Therefore, we were interested in revealing possible roles of insulin, leptin, and prolactin in regulating bovine milk fat synthesis at the transcriptional level.

We hypothesized that hormones and FA could affect the activity of reporters containing response elements for transcription factors involved in regulation of lipid synthesis in bovine

mammary epithelial cells. In our study, luciferase reporter constructs containing response elements for SREBP-1 (4X SRE and SRE complex), PPARγ (3X PPRE), and LXR (3X LXRE) were used in bovine mammary epithelial cells treated with hormones or FA to evaluate their activities. We chose to study the SRE complex and 4X SRE reporter because the complex includes the binding sites of additional transcription factors that might be necessary for transcriptional activation. Additionally, this complex is present in the promoter of SREBP-1 and might reflect that SREBP-1 transcription is regulated by itself in a feed-forward mechanism.

MATERIALS AND METHODS

Transcription Factor Reporter Construction

Oligonucleotides containing overhangs for NheI and XhoI and with tandem transcription factor binding sites, including 4X SRE (Forward: 5'P- ctagatcaccccac atcaccccac atcaccccac atcaccccac; Reverse: 5'P- tcgagtggggtgat gtggggtgat gtggggtgat gtggggtgat); 3X LXRE (Forward: 5'P- ctagcgcccgctagtaaccc tgaccgccagtaaccc cgcccgctagtaaccc; Reverse: 5'P- tcgagggttactagcgggcg gggttactggcggtca gggttactagcgggcg); 3X PPRE (Forward: 5'P- ctagaactgggccaaaggtct aactgggccaaaggtct aactgggccaaaggtct; Reverse: 5'P- tcgaagacctttggcccagtt agacctttggcccagtt agacctttggcccagtt); and an SRE complex containing NFY, Sp1, and SRE sites (Forward: 5'P- ctagtcattggcctgggcggcgcagccaagctgtcagcccatgtggcgtgtcc; Reverse: 5'P- tcgaggacacgccacatgggctgacagcttggctgcgccgcccaggccaatga), were commercially synthesized (Sigma, St. Louis, MO). The oligos for each complex were annealed and then 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.

Cell Culture

Experiments were conducted using the MAC-T bovine mammary epithelial cell line. Cells were maintained in basal culture media consisting of High glucose Dulbecco's Modified Eagle's Medium (Sigma) with 10 kU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and routinely cultured at 37°C in 5% CO_2. For experiments, cells were seeded in 24-well plates at a density of 2×10^4 cells/cm^2. After overnight incubation in basal culture media, cells were treated as described in experiments. Immediately after treatment application, cells were co-transfected with 0.5 μg 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). Each experiment was repeated 4 times and means were calculated.

Hormone Treatment

Hormone media were prepared by supplementing hormones to basal media. Insulin (1 μg/mL; Sigma), prolactin (1 μg/mL, Sigma), or leptin (100 ng/mL, NeoBioScience, Cambridge, MA) was added to basal media. Cells were cultured for 24 h in media containing insulin, prolactin, leptin, or all possible combinations of the three hormones.

Fatty Acid Treatment

Cells were treated with linoleic acid, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA, (Nu-Chek Prep, Inc., Elysian, MN) at 0, 25, 50, 75, or 100 μM. Fatty acids were bound to BSA (Sigma) using the method described by Ip and co-workers (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 μM BSA solution to make a 300 μM BSA-FA salt complex (3:1 FA to BSA molar ratio).

Hormone and Fatty Acid Treatment

Basal medium was removed and replaced with fresh hormone medium containing insulin (1 μg/mL) and leptin (100 ng/mL). *Cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (100 μM) were applied to cells transfected with the SRE complex reporter.

Octadecenoic Acid Isomer Treatment

Cells transfected with reporters containing either SRE complex or 4X SRE were treated with *trans*-10, *cis*-12 CLA, *trans*-11 octadecenoic acid (Nu-Chek), *trans*-10 octadecenoic acid (a gift from Dr. Dale Bauman, Cornell University), *cis*-12 octadecenoic acid (Cayman Chemical, Ann Arbor, MI), or *cis*-9 octadecenoic acid (Nu-Chek) at 100 μM.

Luciferase Assay

After 24 h of treatment and transfection, 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.

Statistical Analysis

All data were analyzed using the General Linear Model (GLM) procedure of SAS (SAS 9.2; SAS Institute, Inc., Cary, NC). Hormone treatment means were compared to the DMEM control using Dunnett's multiple comparison. The intercepts of FA treatments were compared using the solution option in the GLM procedure. Means of hormone × FA and 18:1 FA treatment

were compared using Tukey's multiple comparison procedure. Differences were considered significant when $P < 0.05$.

RESULTS

The insulin and leptin combination increased reporter activity of the SRE complex compared with the untreated cells; however, other hormone treatments did not affect the activity of the reporter (Figure 4.1 A). Activity of the 4X SRE reporter was not changed in any hormone treatments (Figure 4.1 B). For the 3X PPRE reporter, insulin increased luciferase activity (Figure 4.1 C). The 3X LXRE reporter did not respond to any hormone treatments (Figure 4.1 D).

The activity of SRE complex reporter was inhibited by *trans*-10, *cis*-12 CLA compared to linoleic acid and *cis*-9, *trans*-11 CLA (Figure 4.2 A). Both linoleic acid and *trans*-10, *cis*-12 CLA inhibited the activity of 4X SRE reporter compared to *cis*-9, *trans*-11 CLA treated cells (Figure 4.2 B). There was no FA effect observed for 3X PPRE or 3X LXRE reporters (Figure 4.2 C and D).

Because the SRE complex containing reporter activity was inhibited by *trans*-10, *cis*-12 CLA and stimulated by insulin and leptin combination, the interactive effect of insulin and leptin in combination with *trans*-10, *cis*-12 CLA on the SRE complex reporter was tested. In presence of insulin and leptin, *trans*-10, *cis*-12 CLA reduced reporter activity compared to the insulin and leptin treatment or the *cis*-9, *trans*-11 CLA control in presence of insulin and leptin (Figure 4.3).

In order to determine which double bond(s) in *trans*-10, *cis*-12 CLA is inhibitory on reporters containing response elements for SREBP-1, cells were treated with different octadecenoic acid isomers. None of the 18:1 FA had effects on the SRE complex reporter activity (Figure 4.4 A). In contrast, activity of the reporter with 4X SRE insertion was reduced

by *trans*-10, *cis*-12 CLA. Neither *trans*-10 nor *cis*-12 double bond had an inhibitory effect on the 4X SRE reporter activity (Figure 4.4 B).

DISCUSSION

In this study, we examined the effect of hormones, *trans*-10, *cis*-12 CLA, and their interactions on the activation of reporters containing transcription factor response elements and found that hormones are not able to overcome the inhibiting effects of *trans*-10, *cis*-12 CLA on the reporters containing SRE sequences. We also determined that both double bonds in *trans*-10, *cis*-12 CLA were responsible for its inhibitory effect on the reporters. This is the first report revealing the direct effect of FA and hormones on luciferase reporters inserted with response elements for transcription factors involved in lipid synthesis in bovine mammary epithelial cells.

The MFD-inducing role of *trans*-10, *cis*-12 CLA has been well defined and expression of SREBP-1 and lipogenic enzymes was inhibited by infusion of this CLA isomer into dairy cows (Baumgard et al., 2000; Baumgard et al., 2002). Similarly, the proteolytic activation of SREBP-1 was reduced in bovine mammary epithelial cells treated with *trans*-10, *cis*-12 CLA (Peterson et al., 2004). Although the responses of PPARγ and LXR to MFD have not been examined, studies have shown coordinated up-regulation of lipogenic genes when they were 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). To examine the roles of SREBP-1, PPARγ, and LXR in regulating lipid synthesis under the influence of *trans*-10, *cis*-12 CLA, studies are needed to reveal the direct effect of *trans*-10, *cis*-12 CLA on transcription factor activation. In this study, *trans*-10, *cis*-12 CLA reduced the activity of reporters containing SRE, indicating that the reduced lipogenic gene and SREBP-1 expression during MFD observed in other studies is likely directly regulated, at least in part, by

SREBP-1. The lack of response in PPRE or LXRE reporters to *trans*-10, *cis*-12 CLA in the present study does not support roles of PPARγ or LXR in the inhibition of lipogenesis caused by *trans*-10, *cis*-12 CLA. As nuclear factors, PPARγ and LXR can bind to FA and are regulated by FA (Pawar et al., 2002). Liver X receptor also regulates the expression of SREBP-1 and there are binding sites for LXR in the SREBP-1 promoter (Lengi and Corl, 2010; McFadden and Corl, 2010). Previous studies led to our hypothesis that SREBP-1 reduction during MFD is regulated by LXR. However, our results of unchanged LXRE reporter activity by *trans*-10, *cis*-12 CLA do not support our hypothesis that the reduction in SREBP-1 abundance during MFD is regulated by LXR. Instead, evidence suggests that SREBP-1 is regulated through its postive feedback controling pathway.

To further investigate the mechanism of the inhibitory effect of *trans*-10, *cis*-12 CLA on SRE complex and 4X SRE reporter activities, we examined the role of the double bond(s) in mediating the inhibition. Neither *trans*-10 nor *cis*-12 18:1 FA reduced the activity of SRE reporters, indicating that the two double bonds do not exhibit inhibitory effects on SRE reporter activation independently. There is a requirement for the *trans*-10 and *cis*-12 double bonds to be present in a conjugated arrangement. The hypothesized mechanism for *trans*-10, *cis*-12 CLA inhibition is that the proteolytic activation of SREBP-1 is blocked. For the 4X SRE reporter, oleic acid (*cis*-9 18:1) decreased reporter activity, while *trans*-11 18:1 increased it. This is inconsistent with *in vivo* studies showing no change in milk fat synthesis when cows were infused with the two FA (Enjalbert et al., 2000; Griinari et al., 2000). Observations with the 4X SRE reporter from the current study might indicate some artificial effects of *cis*-9 and *trans*-11 18:1 on the reporter activity.

In opposition to the negative relationship between reporter activity and *trans*-10, *cis*-12 CLA, we examined hormones that might activate these reporters. Transition from gestation to lactation coincides with numerous hormonal changes and increased lipid synthetic capacity in the mammary gland. Insulin increased the expression of ACC, the rate-limiting enzyme for *de novo* lipogenesis, in MAC-T cells probably through IGF-I receptor (Kim et al., 1997). In addition, Silva et al. (2002) confirmed the expression of long form leptin receptor in MAC-T cells. Although there is no direct evidence showing prolactin receptor present in MAC-T cells, prolactin stimulated acyltransferase enzyme activities in these cells (Morand et al., 1998).

Previous results with hormones in cultured cells or tissues led us to test insulin, prolactin, and leptin in the current study. Only the SRE complex and 3X PPRE reporters were stimulated by the insulin and leptin combination and insulin alone, respectively. No prolactin effect was observed. Prolactin is an important lactogenic hormone that stimulates milk protein synthesis (Knight, 2001), but our results offer no support for its role in milk fat synthesis. Although insulin promotes lipogenesis in adipose tissue through SREBP-1 in mice (Kim et al., 1998a), it plays little role in bovine mammary lipogenesis (Griinari et al., 1997). Our results showed a stimulatory effect of insulin on PPRE reporter activity and no PPRE response to *trans*-10, *cis*-12 CLA. It might seem that insulin acting through PPARγ could overcome a *trans*-10, *cis*-12 CLA effect; however, milk fat is not influenced by insulin (Griinari et al., 1997). Additional studies are needed to determine a potential regulatory role of PPARγ in mammary lipogenesis. The moderate stimulated SRE complex reporter activity by leptin and insulin in the current study raises the question as to whether the SREBP-1 inhibition by *trans*-10, *cis*-12 CLA could be overcome by insulin and leptin. In order to answer this question, cells transfected with the SRE complex reporter were treated with *trans*-10, *cis*-12 CLA in presence or absence of insulin and

leptin. However, insulin and leptin were unable to rescue the SRE complex reporter activity reduced by *trans*-10, *cis*-12 CLA, indicating no overlap in the systems or a dominant effect of *trans*-10, *cis*-12 CLA on SRE complex containing reporter activity.

In conclusion, we confirmed the inhibitory effect of *trans*-10, *cis*-12 CLA on SRE reporter activities and this inhibition was not modified by insulin and leptin, which showed a stimulatory effect on SRE complex reporter activity. The activity of PPRE reporter was stimulated by insulin and not changed by *trans*-10, *cis*-12 CLA in bovine mammary epithelial cells. Both *trans*-10 and *cis*-12 double bonds have to be present for CLA to exhibit the inhibitory effect on the reporter activity of SRE. This study provides some insight into transcriptional regulation of milk fat synthesis during MFD in dairy cows.

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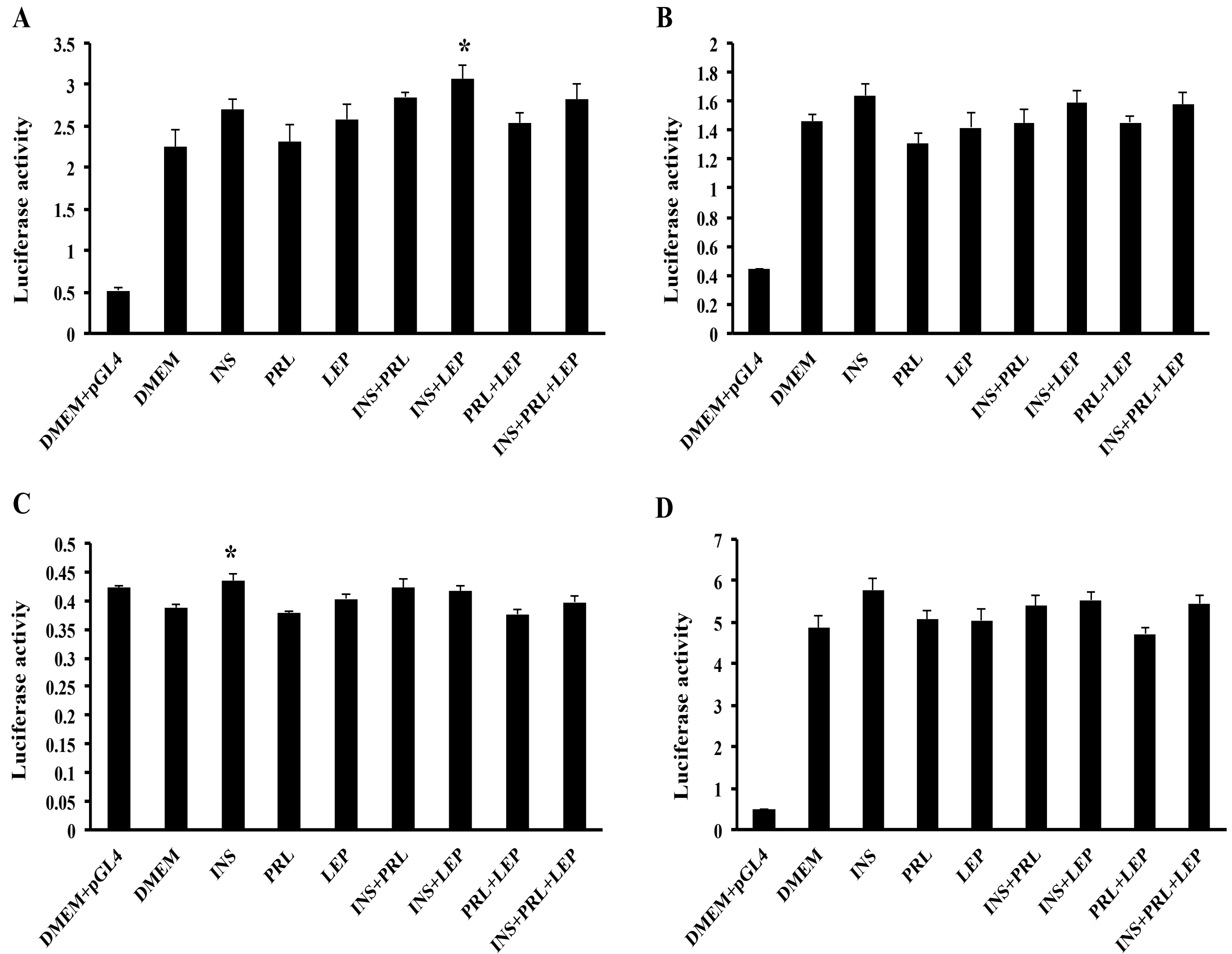
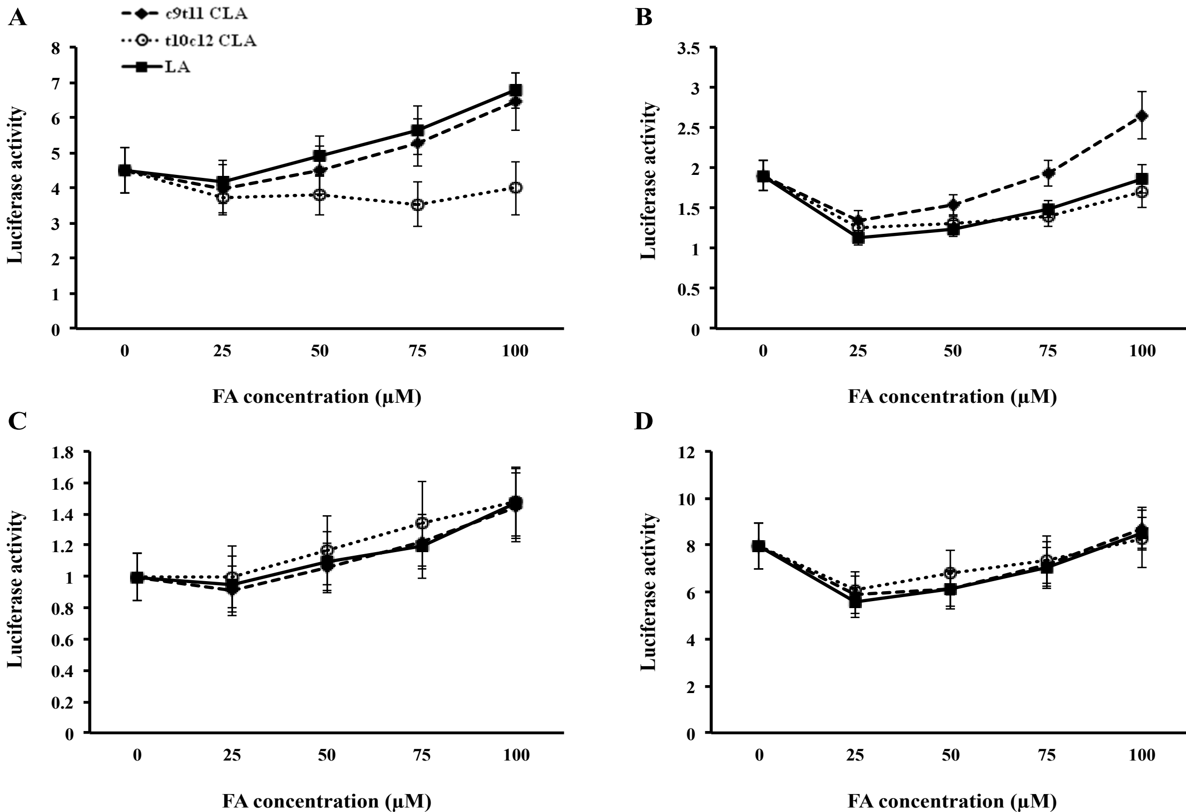
FIGURES

Figure 4.1 The effect of hormones on reporter activities



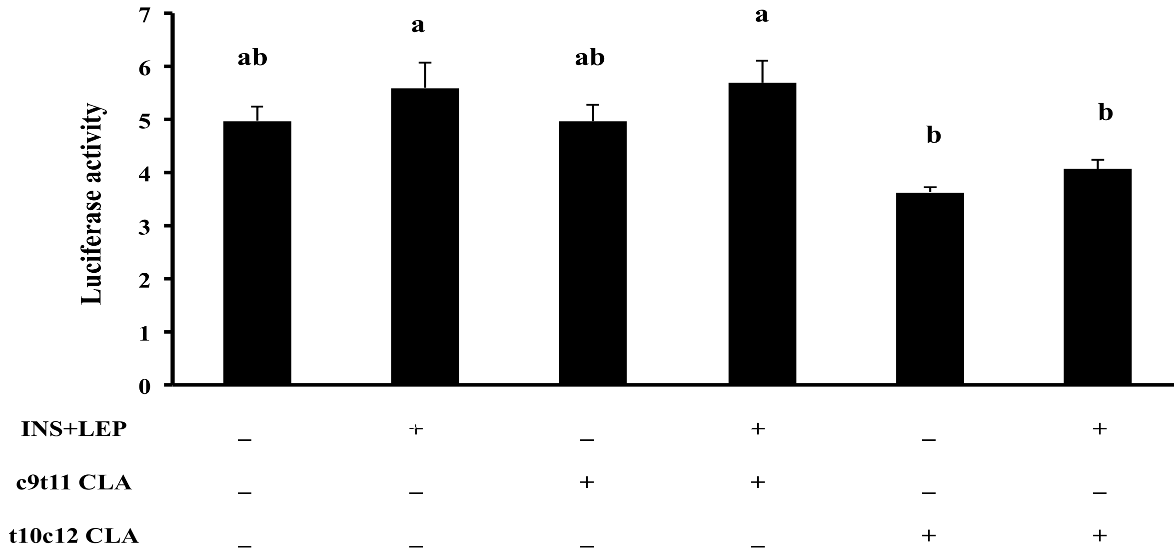
MAC-T cells were treated with insulin (INS, 1 μg/mL), prolactin (PRL, 1 μg/mL), leptin (LEP, 100 ng/mL), or all possible combinations and immediately co-transfected with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 μg/well) containing (**A**) SRE complex, (**B**) 4X SRE, (**C**) 3X PPRE, or (**D**) 3X LXRE in the reporter. 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. The empty pGL4.23[luc2/minP] vector and basal DMEM media were used as transfection and treatment control. Treatment means (n=4) were compared to DMEM control. Difference was considered significant when $P < 0.05$ and indicated by *.

Figure 4.2 The effect of *trans*-10, *cis*-12 CLA on reporter activities



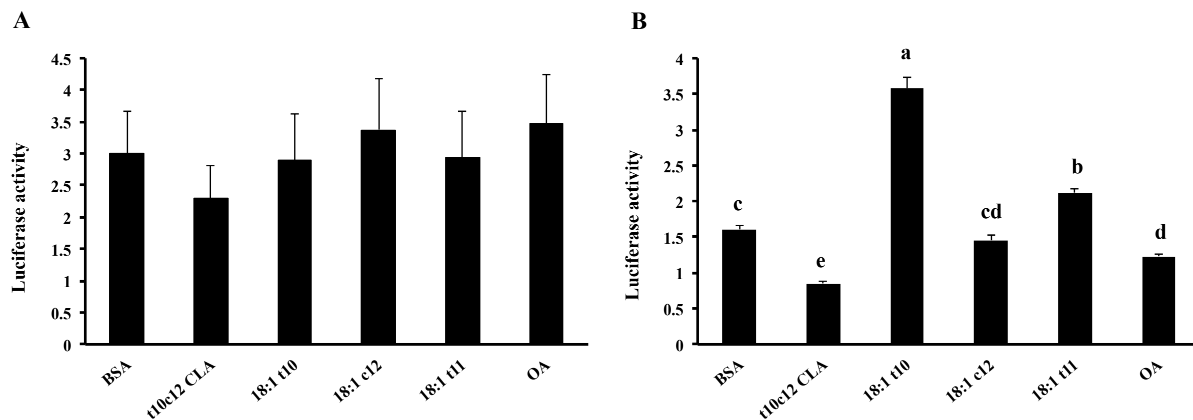
MAC-T cells were treated with linoleic acid (LA), *cis*-9, *trans*-11 CLA (c9t11 CLA), or *trans*-10, *cis*-12 CLA (t10c12 CLA) at 0, 25, 50, 75, or 100 µM, followed by co-transfection with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 µg/well) containing (**A**) SRE complex, (**B**) 4X SRE, (**C**) 3X PPRE, or (**D**) 3X LXRE in the reporter. 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. Data (0 µM excluded, n=4) were plotted using general linear model and slopes were compared. Difference was considered significant when $P < 0.05$.

Figure 4.3 The interactive effect of insulin and leptin combination and *trans*-10, *cis*-12 CLA on reporter activity containing SRE complex



MAC-T cells were treated with *cis*-9, *trans*-11 CLA (c9t11 CLA) or *trans*-10, *cis*-12 CLA (t10c12 CLA) at 100 μM in absence or presence of insulin (1 μg/mL) and leptin (100 ng/mL) (INS+LEP), and immediately co-transfected with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 μg/well) containing SRE complex in the reporter. 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. Basal DMEM media with BSA was used as a control. Treatment means (n=4) were compared. Difference was considered significant when $P < 0.05$ and indicated by different letters.

Figure 4.4 The determination of double bond(s) in *trans*-10, *cis*-12 CLA responsible for inhibited SRE reporter activities



MAC-T cells were treated with *trans*-10, *cis*-12 CLA (t10c12 CLA) or 18:1 FAs at 100 µM, followed by co-transfection with renilla luciferase vector (30 ng/well) and luciferase reporter constructs (0.5 µg/well) containing (**A**) SRE complex or (**B**) 4X SRE in the reporter. 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. Cells treated with BSA were used as a control. Treatment means (n=4) were compared. Difference was considered significant when $P < 0.05$ and indicated by different letters.

Chapter 5

The effect of short-term *trans*-10, *cis*-12 conjugated linoleic acid infusion on milk fat synthesis in lactating dairy cows

INTRODUCTION

Fat is the most variable component in milk and influenced by many factors in lactating dairy cows (Shingfield and Griinari, 2007). Milk fat depression has been observed when cows are fed with diets containing large amounts of readily fermentable carbohydrates/reduced amounts of roughages and diets highly supplemented with unsaturated oils (Bauman and Griinari, 2001). Theories have been proposed to explain MFD, and the most recent one is the biohydrogenation theory that states alteration of ruminal biohydrogenation produces unique FA intermediates that have inhibitory effects on milk fat synthesis (Bauman and Griinari, 2001). *Trans*-10, *cis*-12 CLA has been identified as a major CLA isomer responsible for inhibition of milk fat synthesis (Baumgard et al., 2000) and its role in MFD was confirmed by the progressively reduced milk fat observed with infusion of increasing amounts of *trans*-10, *cis*-12 CLA in dairy cows (Baumgard et al., 2001).

Triacylglycerol comprises more than 95% of the fat in milk (Dils, 1986). Fatty acids for milk fat synthesis come from either *de novo* FA synthesis in epithelial cells (short-chain, medium-chain, and partial 16 carbon FA) or preformed FA originated from dietary lipids or the mobilization of body fat reserves (remaining 16 carbon and longer-chain FA) (Bauman and Griinari, 2001). Fatty acids from both sources were reduced in

dairy cows with diet-induced MFD (Peterson et al., 2003). Many enzymes and proteins are involved in FA biosynthesis, activation, uptake, trafficking, and esterification to glycerol-3-phosphate. The gene expression of enzymes involved in the *de novo* pathway (FAS), FA trafficking and modification (LPL, FABP3 and SCD1), and enzymes associated with triglyceride esterification (GPAT and AGPAT) was reduced during abomasal infusion of *trans*-10, *cis*-12 CLA (Baumgard et al., 2002). Additionally, the expression of SREBP-1, a transcription factor regulating lipid synthesis, was decreased in *trans*-10, *cis*-12 CLA treated bovine mammary epithelial (MAC-T) cells and in cows fed a low forage/high oil diet or infused with *trans*-10, *cis*-12 CLA (Harvatine and Bauman, 2006; Peterson et al., 2004). In addition to SREBP-1, evidence from cell culture studies indicates that PPARγ and LXR might be involved in lipid synthesis in bovine mammary epithelial cells as well (Bionaz and Loor, 2008b; McFadden and Corl, 2010). Thus, these transcription factors might play a role in MFD by regulating lipogenic enzymes.

Our previous studies have shown that some lipogenic enzymes were down-regulated when SREBP-1 was inhibited by siRNA in MAC-T cells (Ma and Corl, 2012). However, we need *in vivo* studies to determine whether SREBP-1, PPARγ and LXR are inhibited during MFD. In the present study, lactating dairy cows were intravenously infused with *trans*-10, *cis*-12 CLA for 12 h to mimic the early stage of MFD where changes start occurring and to examine the activation of transcription factors in dairy cows at the onset of MFD.

MATERIALS AND METHODS

Animals and Treatments

All procedures involving animals were approved by Virginia Tech Institutional Animal Care and Use Committee. Ten paired lactating Holstein cows and 8 paired lactating crossbred cows housed at Virginia Tech dairy farm were randomly assigned to control (**CON**) or *trans*-10, *cis*-12 CLA treatment (**CLA**). Cows were infused with vehicle or 0.625 g/h *trans*-10, *cis*-12 CLA through a jugular catheter. Cows were milked every 4 h starting 12 h before initiation of infusion until the end of infusion. Milk samples were taken at each milking from 0 h to 12 h of infusion for subsequent milk component analysis (United DHIA, Blacksburg, VA) and milk FA composition analysis. The mammary gland was biopsied (Bard Biopsy System, Tempe, AZ) within 2 h of the last milking and infusion of treatments was maintained until completion of the biopsy. A portion of the biopsied mammary tissue was immediately processed in the lab for nuclear protein isolation and the remainder was frozen in liquid nitrogen and subsequently stored at -80°C for RNA and protein analysis. Throughout the experiment, cows had ad libitum access to feed and water.

Infusion Procedure

One or two days before initiation of infusion, a catheter was placed in the jugular vein and maintained with physiological saline (sodium chloride 0.9% injection USP bag; Baxter, Deerfield, IL) and 100 IU/mL of heparin (Hospira, Inc., Lake Forest, IL). *Trans*-10, *cis*-12 CLA was infused as an emulsified FA mixture containing 60% CLA isomers with equal amounts of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA as free FA (BASF,

Ludwigshafen, Germany). The infusion volume was adjusted to provide 0.625 g/h of *trans*-10, *cis*-12 CLA. A vehicle infusion at the same rate was used as the control.

Emulsion Preparation

Emulsions were prepared as described by Mashek et al. (2005). Briefly, 500 ppm of ethoxyquin (MP Biomedicals, Santa Ana, CA) was added to the CLA oil. In separate beakers, 34 g of CLA oil and 130 mL of water were heated to 70°C. Two grams of lecithin (Acros Organics, Geel, Belgium) was added to the water and the mixture was homogenized using a hand blender (Oster, 2609) for 1 min. The heated lipid was added to the lecithin and water mixture, followed by glycerol (8.5 g; Sigma, St. Louis, MO) and mixed for 2 min using the blender. Subsequently, the coarse emulsion was passed through an APV 15MR homogenizer (SPX Corporation, Delavan, WI), 500 psi for the first stage and 2000 psi for the second stage. The emulsion pH was adjusted to 8.3 using 1 N NaOH and then it was autoclaved for 25 min at 121°C and 21 psi. After sterilization, the emulsion was cooled at 4°C overnight and the contents were transferred to a sterile 1000-mL saline bag (Baxter) and stored at 4°C until use. To prepare the vehicle control, CLA oil was replaced with water to reach an equal volume.

Fatty Acid Analysis

For milk FA analysis, lipid extraction from milk fat was performed according to Hara and Radin (1978) and described by Chouinard et al. (1999b). Fatty acids in extracted lipid were then transesterified with sodium methoxide according to Christie (1982) with modifications described by Kelsey et al. (2003).

Fatty acid methyl esters were analyzed by gas chromatography (Agilent 6890N GC) using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d. with 0.2 μm thickness;

Varian, Inc., Palo Alto, CA). Run conditions were as follows: the oven temperature was initially set at 70°C, then increased at 8°C/min to 110°C, then increased at 5°C/min to 170°C and held for 10 min. The inlet and detector temperatures were 250°C, the split ratio was 100:1, and a 1 µL injection volume was used. The hydrogen carrier gas flow rate was 1 mL/min. Hydrogen flow to the detector was 25 mL/min, airflow was 400 mL/min, and the flow of nitrogen makeup gas was 40 mL/min. Fatty acid peaks were identified by using pure methyl ester standards (Nu-Check Prep Inc., Elysian, MN).

Real-Time PCR

Total RNA was extracted from frozen tissue using RNAzol RT (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer’s instructions. After extraction, ribonucleic acid pellets were resuspended in RNase-free water and concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). RNA (1 µg per reaction) was reverse transcribed to complementary DNA using the Omniscript RT kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Oligo-dT (Eurofins MWG/Operon, Huntsville, AL) was used as the primer for reverse transcription. Real-time quantitative PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI) in an Applied Biosystems 7300 Real-time PCR machine (Applied Bisystems, Foster City, CA). Reactions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. Each reaction was performed in duplicate wells. Gene-specific primers for the transcripts used in the study are shown in Table 5.1.

Immunoblotting

Frozen tissues were homogenized in cold lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA pH 8.0, and mammalian protease inhibitor cocktail (Sigma)), followed by centrifugation at 16,100 ×*g* for 15 min at 4°C. Supernatants were collected for protein concentration measurements, using the Bradford assay (Bio-Rad, Hercules, CA). To ensure equal loading, samples were diluted to the same protein concentrations with Laemmli sample buffer (Sigma) and heated at 95°C for 10 min. Proteins were separated by electrophoresis using polyacrylamide gels (12%; Lonza, Basel, Switzerland) and transferred to a PVDF membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were then blocked in blocking buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween, and 5% dried non-fat milk) on a rocker for 1 h at room temperature. Membranes were probed with primary anti-SREBP-1 antibody (SC-13551, Santa Cruz Biotechnology, Santa Cruz, CA) or primary anti-SCD1 antibody (custom rabbit anti-bovine SCD1, Pacific Immunology, Ramona, CA) at 1:1000 or 1:2000 in blocking buffer at 4°C overnight. Membranes were washed in washing buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, and 0.1% Tween) twice for 10 min. Following washing, membranes were incubated with horseradish peroxidase-conjugated goat, anti-mouse IgG1 or anti-rabbit secondary antibody (Santa Cruz Biotechnology) at 1:1000 or 1:2000 in blocking buffer for 1 h at room temperature. Membranes were washed three times, for 15 min each, and proteins were detected using ECL-Plus chemiluminescence substrate (Amersham Biosciences, Pittsburg, PA) according to manufacturer's instructions. Chemiluminescence was measured using a Chemidoc XRS digital imaging

system and densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay (EMSA)

Mammary tissues were transported to the lab in ice cold PBS immediately after biopsies. Tissues (100 – 200 mg) were chopped into small pieces in ice cold PBS buffer. Following centrifugation at 200 × *g* for 5 min, PBS was removed and nuclear protein was extracted from the pellet using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo, Rockford, IL) according to the manufacturer's instructions. Subsequently, the nuclear extract was dialyzed in 250 mL of dialysis media (10 mM Tris HCl, 50 mM KCl, 1 mM dithiothreitol (DTT), and pH 7.2) for 15 min. Protein concentrations were determined by Bradford assay (Bio-Rad) and samples were aliquoted to 20-25 µL followed by storage at -80°C.

Electrophoretic mobility shift assay was performed using a chemiluminescent EMSA kit (Thermo Fisher) according to the manufacturer's protocol. Briefly, a 6% polyacrylamide gel was poured the day before the assay. While the gel was pre-run at 150 V, biotin end-labeled duplex DNA (Sigma), containing response elements for transcription factors, was incubated with the nuclear extract for 20 min. Following addition of 5X loading buffer, the reaction was electrophoresed at 150 V for 2 h on the gel. The DNA was then transferred to a nylon membrane (Whatman, Maidstone, UK) at 150 mA for 4 h and crosslinked to the membrane using a FB-UVXL-1000 UV CrossLinker (Thermo Fisher). Biotin end-labeled probes were detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher) according to the manufacturer's instructions. Briefly, the membrane was blocked in the blocking buffer

for 15 min with gentle shaking, followed by incubation with streptavidin-Horseradish Peroxidase Conjugate for 15 min. After washing, membranes were incubated with the substrate for 5 min. Chemiluminescence was measured using a Chemidoc XRS digital imaging system and densitometry was performed using Quantity One software (Bio-Rad). Competition with unlabeled probes was used as a control to verify specificity of DNA-protein interactions.

Statistical Analysis

Real-time PCR data were normalized using the geometric mean of eukaryotic translation initiation factor 3 subunit K (EIF3K) and ribosomal protein S15 (RPS15) which were calculated using geNorm software (Bionaz and Loor, 2007; Vandesompele et al., 2002). The desaturase index was calculated for four pairs of FA that represent products and substrates for SCD1. These FA pairs were *cis*-9 14:1/14:0, *cis*-9 16:1/16:0, *cis*-9 18:1/18:0, and *cis*-9*, trans*-11 CLA/*trans*-11 18:1. We defined the desaturase index as follows: [product of desaturase]/[product of desaturase + substrate of desaturase]. The total FA desaturation was calculated by the combination of the 4 FA pairs.

Data for milk FA composition, desaturase index, real-time PCR, western immunoblotting and EMSA were analyzed using the General Linear Model of SAS, while milk and milk component yield were analyzed using the Glimmix procedure (SAS 9.2; SAS Institute Inc., Cary, NC). The model for Glimmix procedure included the fixed effects of treatment, time, and treatment by time interaction. Differences were considered significant when $P < 0.05$.

RESULTS

The yield of milk and milk components was not changed by *trans*-10, *cis*-12 CLA infusion (Figure 5.1). *Trans*-10, *cis*-12 CLA appeared in the milk from CLA cows, but not control cows. The content of 4:0, 6:0, 18:0, *trans* 6-8, 18:1 and C20:0 in milk fat was increased by the CLA treatment, while 14:1 and 15:0 content in milk fat was decreased (Table 5.2). *De novo* synthesized or preformed FA were not affected by the CLA treatment compared to control (Figure 5.2 A). *Trans*-10, *cis*-12 CLA decreased the desaturase index for 18:0 ($P < 0.01$), but did not affect desaturation of other FA or total desaturation, with a trend for reduced 14:0 desaturation ($P = 0.07$; Figure 5.2 B-F).

The mRNA levels for SREBP-1 and PPARγ were reduced by 30 and 20%, respectively, in cows infused with *trans*-10, *cis*-12 CLA, but not significantly ($P = 0.18$ and 0.34; Figure 5.3 A and B). The mRNA expression of the two LXR isoforms, α or β, was not changed by the CLA treatment compared to the control (Figure 5.3 C and D). In addition to the transcription factors, a 30% reduction in both SCD1 and FAS, two representative targets for the transcription factors, was observed for cows treated with *trans*-10, *cis*-12 CLA, but not significantly ($P = 0.33$ and 0.31; Figure 5.3 E and F).

Neither the precursor nor the mature form of SREBP-1 was affected during *trans*-10, *cis*-12 CLA infusion (Figure 5.4). Similarly, the CLA treatment did not have any effect on the protein level of SCD1 (Figure 5.5).

The binding activity of PPARγ was somewhat reduced during *trans*-10, *cis*-12 CLA infusion, but not significantly ($P = 0.7$; Figure 5.6 A). No change was observed for LXR binding to DNA in *trans*-10, *cis*-12 CLA treatment compared to the control (Figure 5.6 B).

DISCUSSION

The role of *trans*-10, *cis*-12 CLA in inhibiting milk fat synthesis during diet-induced MFD has been well defined. Abomasal infusion of 10 g/d *trans*-10, *cis*-12 CLA for 4 d in dairy cows resulted in 42 and 44% reduction in milk fat percentage and yield, respectively (Baumgard et al., 2000). To determine the dosage of *trans*-10, *cis*-12 CLA needed to induce MFD, Baumgard et al. (2001) carried out a study abomasally infusing dairy cows with *trans*-10, *cis*-12 CLA for 5 d at different doses, and concluded that milk fat was markedly inhibited by CLA infusion at 3.5 g/d and was progressively reduced with increasing amounts of *trans*-10, *cis*-12 CLA. Beyond dosage, the time course response of milk fat synthesis to CLA treatment is also critical for studying the mechanism of MFD. Most CLA infusion studies lasted 4 or 5 d to reach a nadir in milk fat synthesis before tissue sample collection. It is necessary to investigate the changes of milk fat synthetic pathways during early stages of MFD to better understand MFD mechanisms. Milk fat percentage was shown to decrease progressively after 2 h of abomasal infusion of *trans*-10, *cis*-12 CLA and became significant after 14 h of infusion (Harvatine and Bauman, 2011). However, the responses of lipogenic genes and their regulators were not investigated in that study. The objective of the current study was to examine the activation of transcription factors at an early stage of MFD; therefore we selected a short time period (14 h) to examine the changes in transcription factor activation and lipogenic enzymes in an effort to reduce the likelihood that any consequential effects of chronic MFD would be detected.

We chose 14 h as the time point to examine transcription factors in milk fat synthesis, based on the fact that SREBP-1 is involved in regulation of bovine mammary

lipogenesis during MFD and the half lives for SREBP-1 mRNA and protein are about 10 and 3 h, respectively (Hirano et al., 2001; Xu et al., 2001). Compared with transcription factors, the mRNA half lives for other genes are generally longer (Sharova et al., 2009). Thus, transcription factor activation levels might be reduced at 14 h of CLA infusion without any possible effects from chronic MFD, so that the roles of transcription factors in MFD can be better defined. Unchanged milk fat yield in this study indicated no overt MFD occurred by 12 h of infusion. Although there were changes in milk FA composition, FA from *de novo* synthesis were not affected by the treatment, further indicating no changes in milk fat synthesis in agreement with no change in FAS gene expression. As a substrate for SCD1, stearic acid (18:0) desaturation was reduced in the CLA treated cows. However, the desaturation of other milk FA was not affected by the CLA treatment, consistent with the unchanged SCD1 gene and protein expression. The activity of SCD, however, might be affected by the treatment. As a major substrate, desaturation of stearic acid might be more sensitive to inhibition by *trans*-10, *cis*-12 CLA.

Our previous study showed that SREBP-1 regulated lipid synthesis by controlling the expression of lipogenic enzymes in MAC-T bovine mammary epithelial cells (Ma and Corl, 2012). Therefore, we intended to further determine its role in regulating milk fat synthesis *in vivo*. In the present study, the 33% reduction observed for SREBP-1 mRNA was not significant. A study using MAC-T cells reported that the mRNA and precursor protein levels of SREBP-1 were not affected but the nuclear, active SREBP-1 was decreased when treated with 75 μM *trans*-10, *cis*-12 CLA for 48 h, indicating that *trans*-10, *cis*-12 CLA reduced lipid synthesis in MAC-T cells by inhibiting the proteolytic activation of SREBP-1 and therefore the transcription of lipogenic genes under its

regulation (Peterson et al., 2004). Harvatine and Bauman (2006) also reported decreased mRNA expression of SREBP-1 in MFD cows induced by a low forage/high oil diet or intravenous infusion of 10 g/d *trans*-10, *cis*-12 CLA for 3 d. However, the lasting time of 14 h and undetectable MFD in the present study might explain why SREBP-1 mRNA or protein expression were not changed by *trans*-10, *cis*-12 CLA.

The expression of PPARγ was increased in the mammary gland with the transition to lactation, indicating a potential role of this transcription factor in regulating milk fat synthesis (Bionaz and Loor, 2008b). The effect of *trans*-10, *cis*-12 CLA on PPARγ expression has been examined using 3T3 cells and PPARγ expression was reduced with *trans*-10, *cis*-12 CLA treatment, implying an inhibitory effect of CLA on PPARγ expression (Kang et al., 2003). Kadegowda et al. (2009) reported that PPARγ expression was not changed by culturing bovine mammary epithelial cells with *trans*-10, *cis*-12 CLA, but some lipogenic genes were up-regulated by a PPARγ agonist, indicating a possible role of PPARγ in lipid synthesis regulation through its activation in these cells. Because PPARγ belongs to the nuclear receptor family and FA can be its ligands, we hypothesized that *trans*-10, *cis*-12 CLA might reduce lipogenic gene expression by inhibiting PPARγ activity. In our current study, mRNA level and DNA-binding activity of PPARγ were decreased by about 19 and 33%, respectively, but no significant differences were observed, probably due to undetectable MFD. It has been reported that *trans*-10, *cis*-12 CLA antagonized ligand-dependent PPARγ activity, possibly via PPARγ phosphorylation, in cultured human primary adipocytes (Kennedy et al., 2008). Whether phosphorylation plays a role in PPARγ-mediating MFD has not been investigated.

The two isoforms of LXR, α and β, have different tissue distributions and both regulate lipid metabolism (Peet et al., 1998). As a nuclear factor, activation of LXR by its agonist, without changing mRNA abundance, increased *de novo* FA synthesis and the expression of SREBP-1 and FAS in cultured bovine mammary epithelial cells, demonstrating a possible role of LXR in lipid synthesis in the cells (McFadden and Corl, 2010). There is also evidence showing unchanged LXR expression during MFD induced by diet or *trans*-10, *cis*-12 CLA in dairy cows (Harvatine and Bauman, 2007). Thus, LXR might play a role in regulating milk fat synthesis through its activation instead of its expression. In the current study, neither LXR mRNA level nor its DNA-binding activity were changed with CLA treatment. Studies with overt MFD are needed to investigate the responses of LXR expression and activity to MFD.

The previous short-term *trans*-10, *cis*-12 CLA abomasal infusion study used a high initiating dose (7.5 g), followed by 2.5 g every 4 h (Harvatine and Bauman, 2011). In contrast, *trans*-10, *cis*-12 CLA was constantly infused at 2.5 g/4 h in the current study, which might have led to a lag time for CLA acting on the mammary gland. Therefore, the time for *trans*-10, *cis*-12 CLA concentration at a steady state in the mammary gland might be less than 12 h. Based on the half lives of mRNA and protein, the expression of transcription factors and their targets was unlikely to be changed by the treatment. In addition, *trans*-10, *cis*-12 CLA uptake (0.05 g/100 g FA) in the current study was not as great as another short-term study in which milk fat was reduced significantly by 14 h of abomasal CLA infusion (~ 0.65 g/100 g FA) (Harvatine and Bauman, 2011). Thus, the shorter time and less *trans*-10, *cis*-12 CLA uptake might be the reason for unchanged transcription factor expression and activity. Additionally, abomasal infusion differs from

intravenous infusion. Abomasal infusion allows absorption by the digestive tract and esterification by the liver, so that the processing of infused *trans*-10, *cis*-12 CLA might influence its effect on the mammary gland. In the current study, the somatic cell count in CLA treatment cows (1,781,000) was significantly higher than that in control cows (241,000; data not shown), indicating possible udder health issues in treatment cows. Therefore, one possibility for the insignificant results is that the health issues of the mammary gland interfered with the action of *trans*-10, *cis*-12 CLA. Cows from different breeds might differ in responding to *trans*-10, *cis*-12 CLA infusion, another possible explanation for the discrepancy between previous studies and ours, but we need more studies to confirm it.

In spite of the different infusion procedure, mammary health issues, and possible breed effect, the trend of decreased mRNA expression of SREBP-1 and PPARγ, and reduced protein-DNA binding for PPARγ in the current study might give us some insight on transcriptional regulation of milk fat synthesis during MFD. Another significant finding of the current study is the demonstration of direct DNA binding for PPARγ and LXR in bovine mammary gland, providing a possibility for the transcriptionally regulatory role of PPARγ and LXR in milk fat synthesis.

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TABLES

Table 5.1 Primer sequences for real-time PCR

Gene	Accession number		Primers (5'-3')
SREBP-1	NM_001113302	Forward	atgccatcgagaaacgctac
		Reverse	gtccgcagactcaggttctc
PPARγ	NM_181024	Forward	catcttccaggggtgtcagt
		Reverse	tcctaccccaggagtatagg
LXRα	NM_001014861	Forward	gaccgactgatgttcccaag
		Reverse	acagaagacacggaggagga
LXRβ	NM_001014883	Forward	tgttgtcttacacccgcatc
		Reverse	cagtcccttcctaccccagt
FAS	NM_001012669	Forward	ctgcaactcaacgggaactt
		Reverse	aggctggtcatgttctccag
SCD1	AY241933	Forward	ccctttccttgagctgtctg
		Reverse	atgctgactctctcccctga
EIF3K	NM_001034489	Forward	gcgatgtttgagcagatgag
		Reverse	gcattttctttggcctgtgt
RPS15	XM585783	Forward	ctctgtgcattcgggttttc
		Reverse	gggctctctgggttcctct

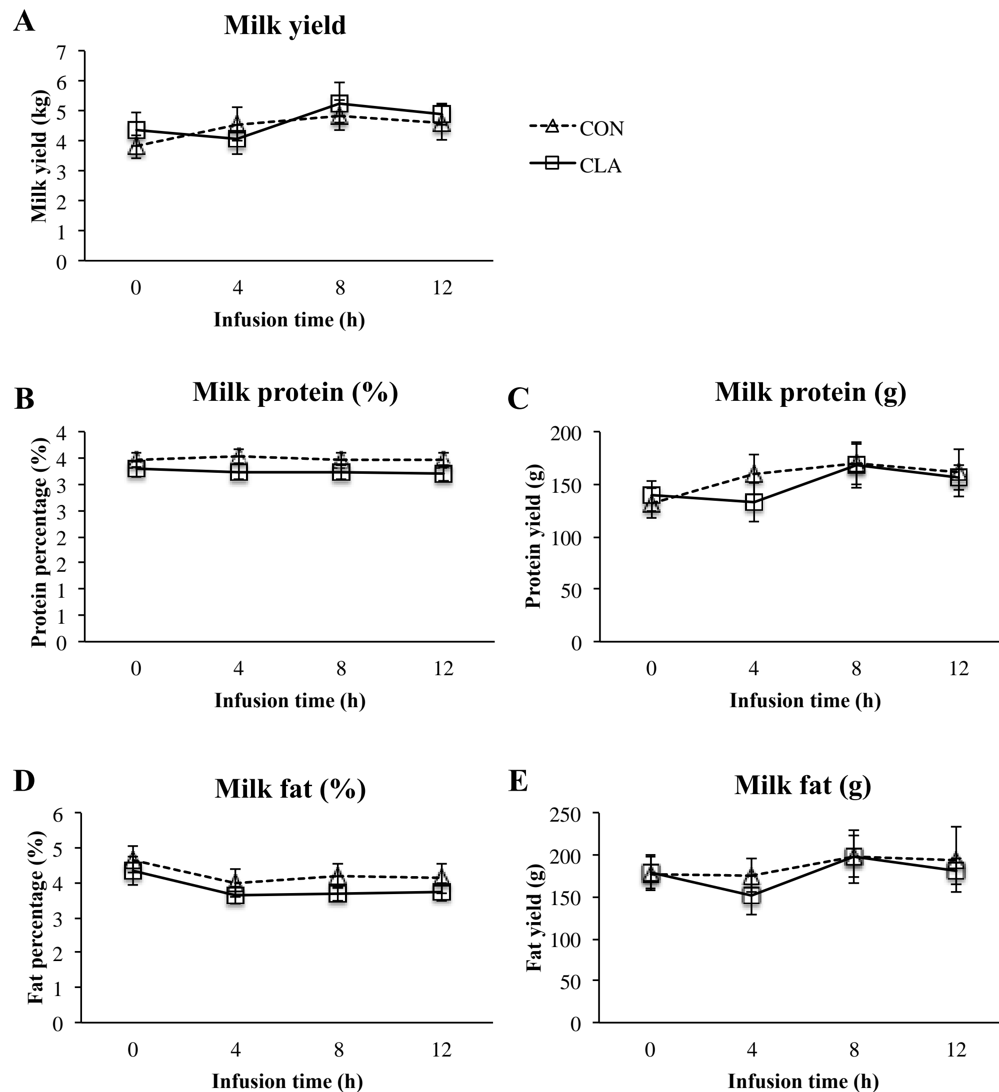
Table 5.2 Milk fatty acid composition

Fatty acids (g/100 g FA)	Treatments CON[a]	CLA[b]	SEM[1]
4:0	3.05	3.37*	0.09
6:0	1.73	1.88*	0.05
8:0	1.11	1.15	0.03
10:0	2.93	2.89	0.09
12:0	3.64	3.49	0.12
14:0	9.99	9.62	0.20
14:1	0.95	0.80*	0.04
15:0	0.86	0.77**	0.02
16:0	29.05	28.63	0.46
16:1	1.70	1.83	0.07
17:0	0.60	0.57	0.01
18:0	10.61	11.71**	0.27
Trans-6-8, 18:1	0.33	0.36*	0.01
Trans-9, 18:1	0.28	0.29	0.01
Trans-10, 18:1	0.60	0.64	0.04
Trans-11, 18:1	0.95	1.06	0.05
Trans-12, 18:1	0.54	0.56	0.02
Cis-9, 18:1	24.48	23.55	0.53
Cis-9, *cis*-12 18:2	2.90	3.03	0.11
20:0	0.12	0.14**	0.00
18:3	0.27	0.28	0.01
Cis-9, *trans*-11 CLA	0.45	0.51	0.02
Trans-10, *cis*-12 CLA	0.00	0.05**	0.01
Others	2.87	2.81	0.05

Treatment means are least square means.
[a]Vehicle control
[b]*Trans*-10, *cis*-12 CLA
[1]Standered error of the mean
*$P < 0.05$; **$P < 0.01$

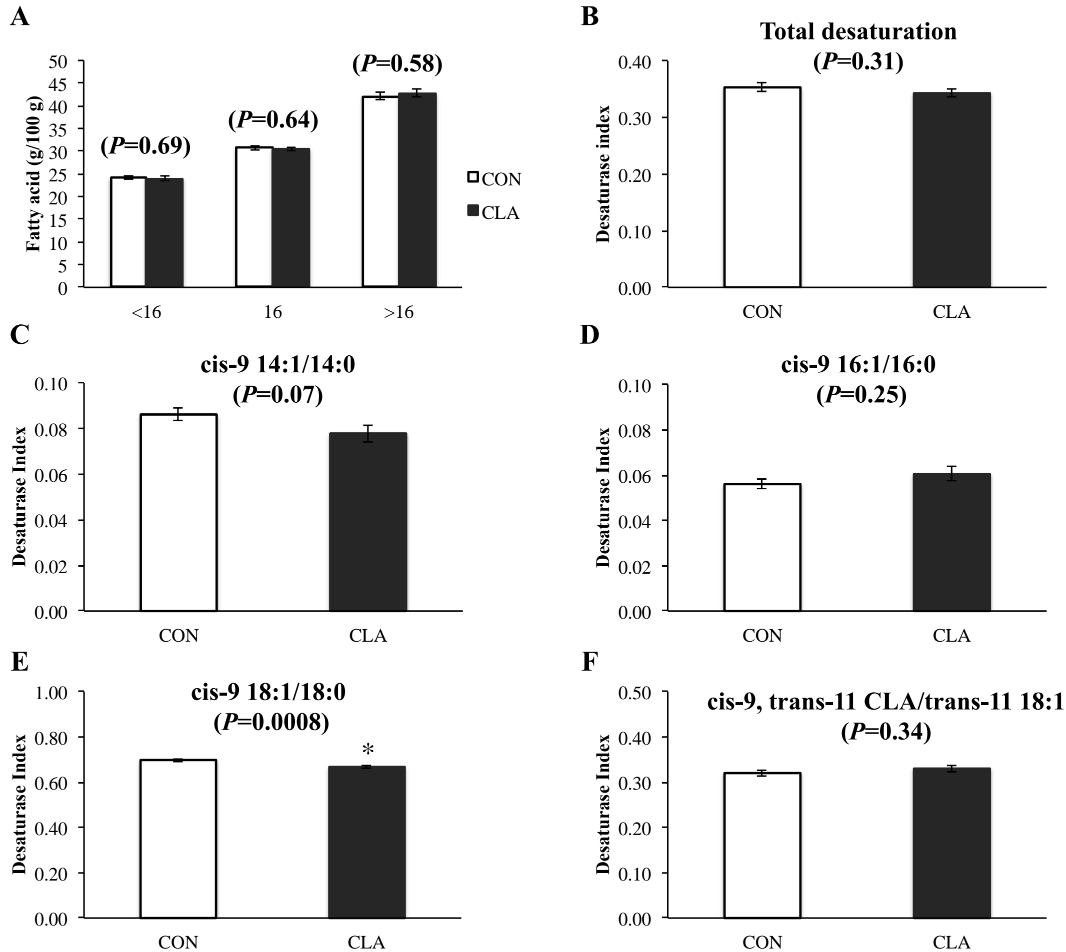
FIGURES

Figure 5.1 The yield of milk, milk protein, and milk fat



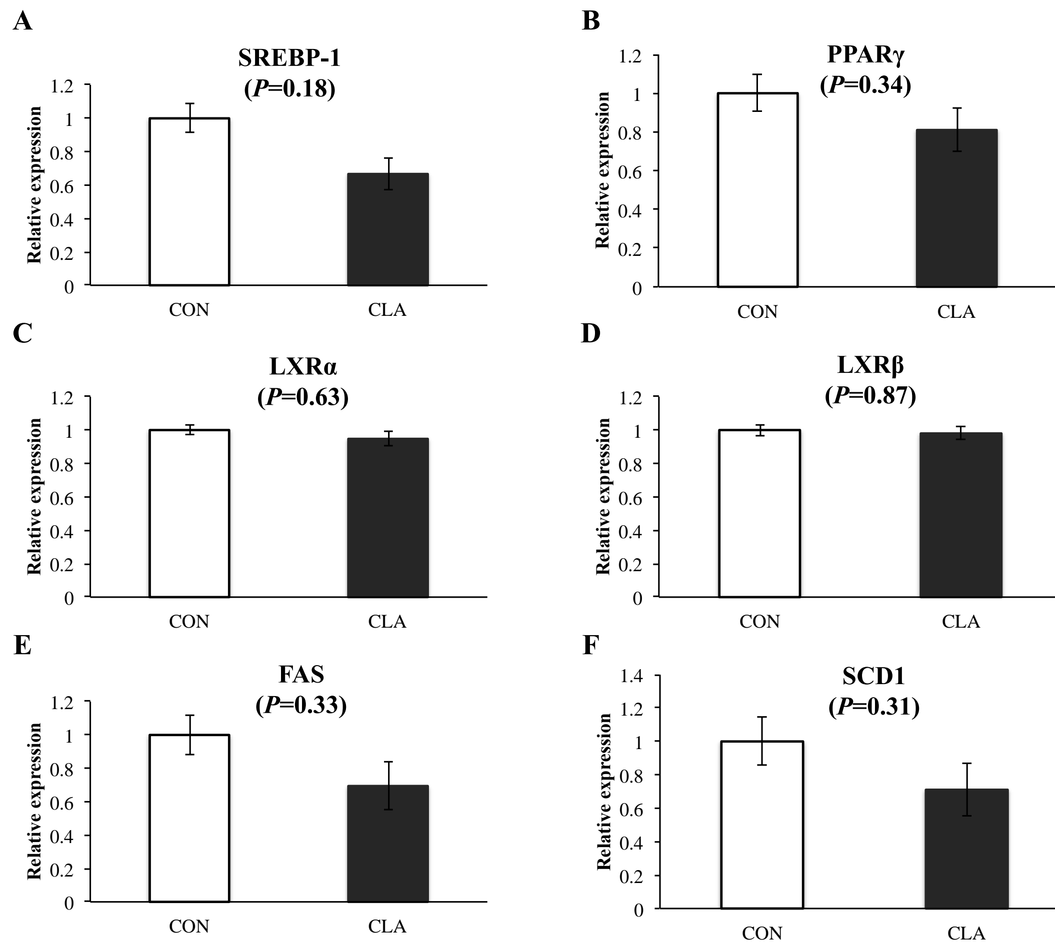
Lactating dairy cows were intravenously infused with either a vehicle control (CON) or 15 g/d *trans*-10, *cis*-12 CLA (CLA) for 12 h. Cows were milked every 4 h during infusion. (**A**) Milk yield was recorded and milk samples were collected for each milking. The (**B,C**) protein and (**D,E**) fat content of milk were analyzed and means for each milking were compared between CON and CLA. Difference was considered significant when $P < 0.05$.

Figure 5.2 Milk FA production from different sources and desaturation



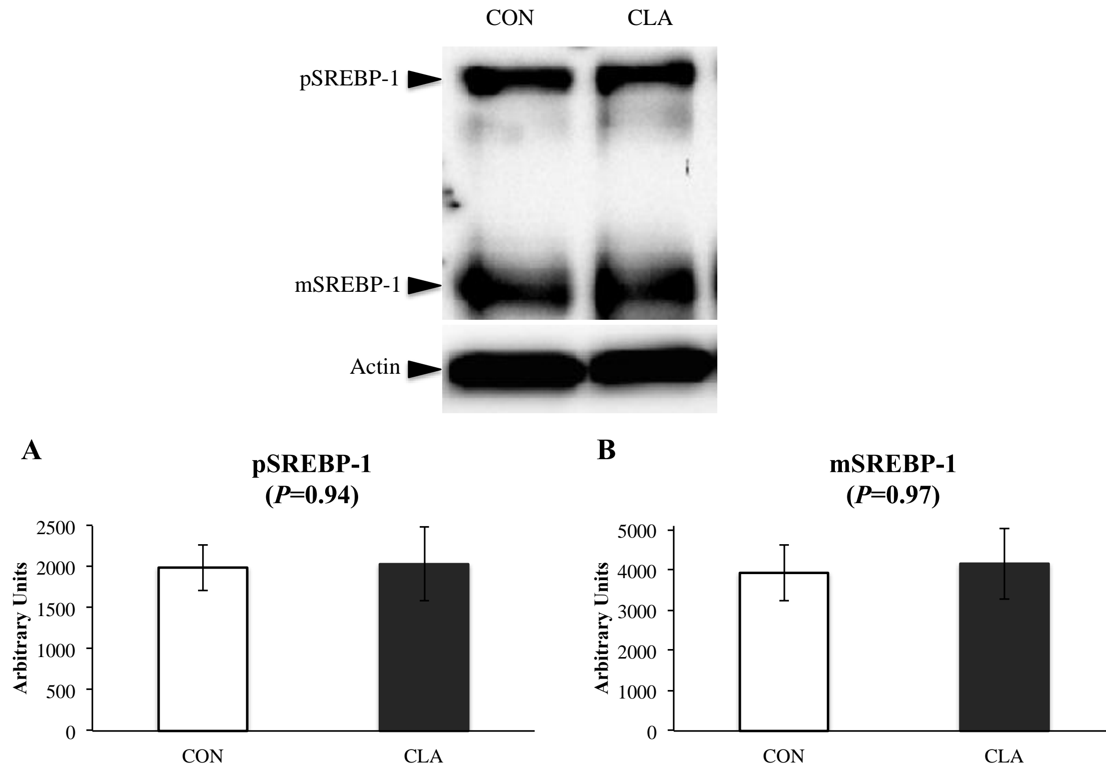
Lactating dairy cows were intravenously infused with either a vehicle control (CON) or 15 g/d *trans*-10, *cis*-12 CLA (CLA) for 12 h. Cows were milked every 4 h during infusion. Milk samples were collected for each milking. Milk FA composition was analyzed by gas chromatography. (**A**) Milk FA were grouped by sources, including *de novo* synthesized FA (<16 carbons), preformed FA (>16 carbons), and both sources (16 carbons). The desaturase index was calculated using the equation: [product of desaturase]/[product of desaturase + substrate of desaturase]. Four pairs of FA that represent products and substrates for SCD1 were used for the calculation. These FA pairs were (**C**) *cis*-9 14:1/14:0, (**D**) *cis*-9 16:1/16:0, (**E**) *cis*-9 18:1/18:0, and (**F**) *cis*-9*, trans*-11 CLA/*trans*-11 18:1. (**B**) Total desaturation was the combination of above 4 FA pairs. Means were compared between CON and CLA. Difference was considered significant when $P < 0.05$ and indicated by *.

Figure 5.3 The mRNA expression of SREBP-1, PPARγ, LXRs and their representative target genes, FAS and SCD1



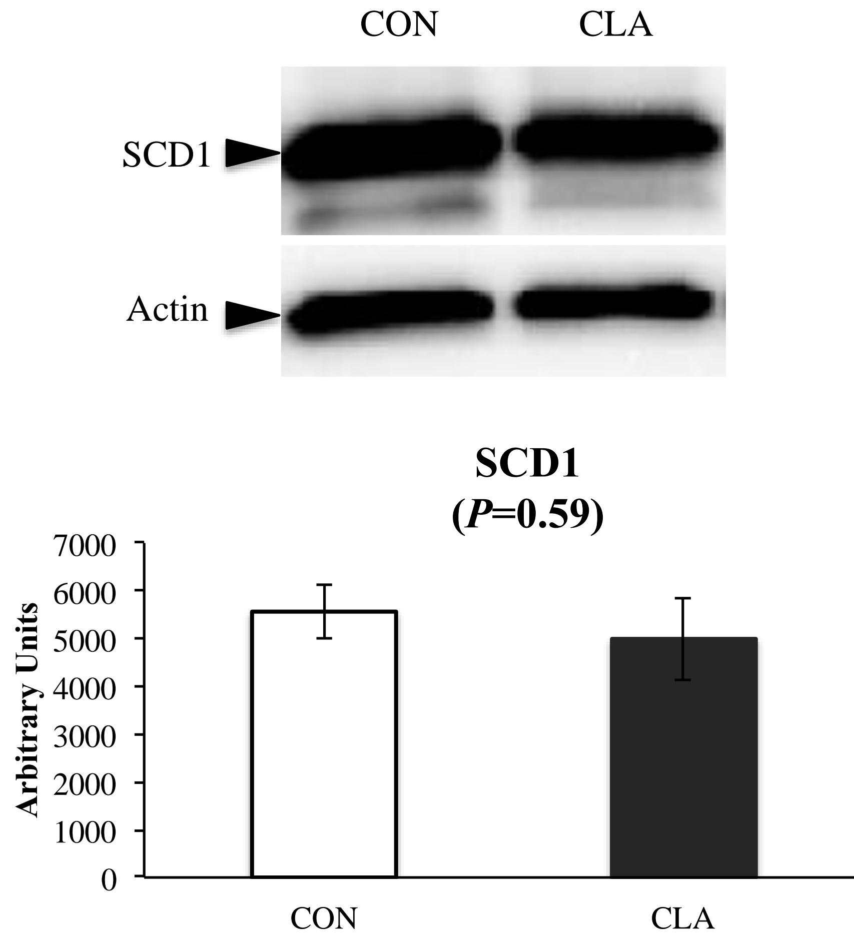
Lactating dairy cows were intravenously infused with either a vehicle control (CON) or *trans*-10, *cis*-12 CLA (CLA) for 12 h, followed by mammary biopsies. Mammary tissues were frozen in liquid nitrogen and total RNA was extracted. RNA was reverse transcribed to cDNA and real-time PCR was performed using corresponding primers. Data were normalized using the geometric mean of EIF3K and RPS15. Means were compared between CON and CLA. Difference was considered significant when $P < 0.05$.

Figure 5.4 Protein abundance of precursor and mature SREBP-1



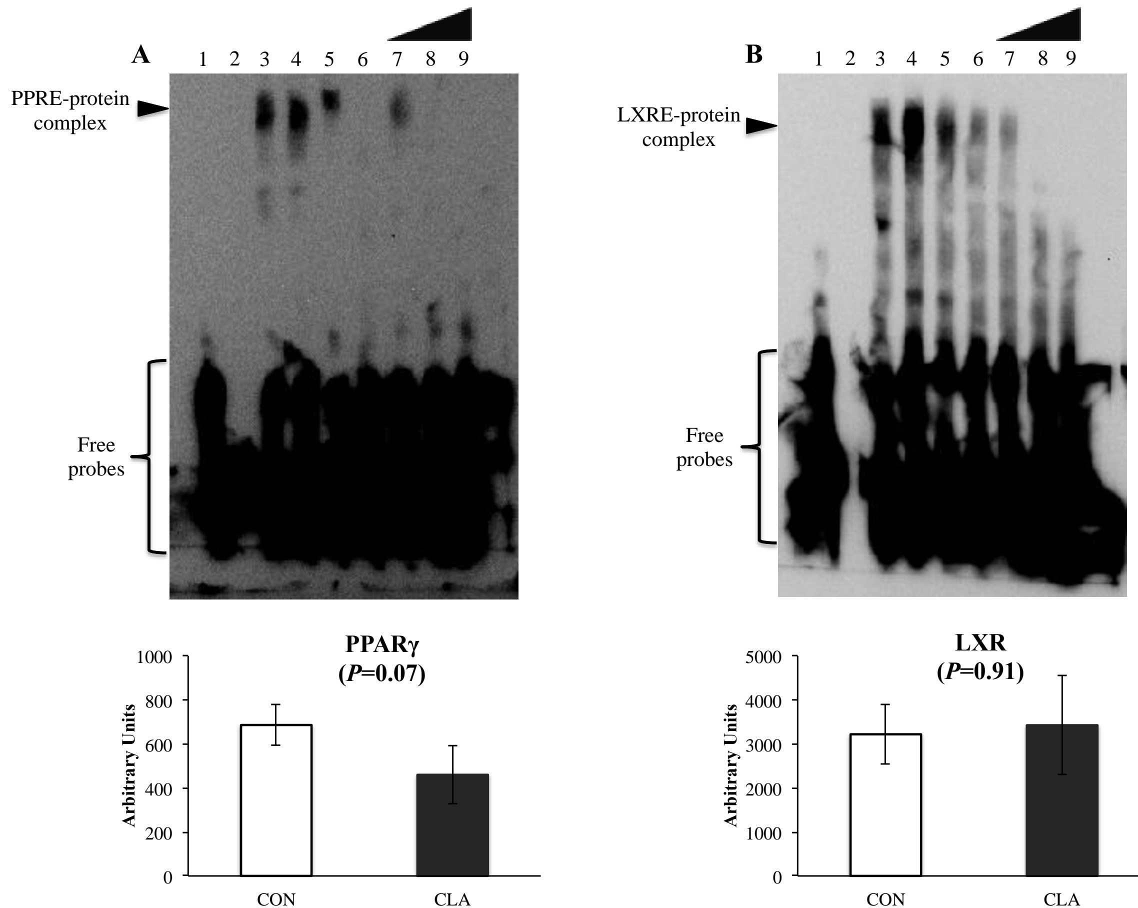
Lactating dairy cows were intravenously infused with either a vehicle control (CON) or *trans*-10, *cis*-12 CLA (CLA) for 12 h, followed by mammary biopsies. Proteins were extracted from mammary tissues and separated by electrophoresis. SREBP-1 proteins were detected using a specific antibody for SREBP-1. Precursor and mature protein bands were visualized and quantified. β actin was used as a loading control. Means were compared between CON and CLA. Difference was considered significant when $P < 0.05$.

Figure 5.5 Protein abundance of SCD1



Lactating dairy cows were intravenously infused with either a vehicle control (CON) or *trans*-10, *cis*-12 CLA (CLA) for 12 h, followed by mammary biopsies. Proteins were extracted from mammary tissues and separated by electrophoresis. SCD1 protein was detected using a specific antibody for SCD1. β actin was used as a loading control. Protein bands were visualized and quantified. Means were compared between CON and CLA. Difference was considered significant when $P < 0.05$.

Figure 5.6 Electrophoretic mobility shift assay for PPARγ and LXR



Lactating dairy cows were intravenously infused with either a vehicle control (CON) or *trans*-10, *cis*-12 CLA (CLA) for 12 h, followed by mammary biopsies. Nuclear proteins were extracted from mammary tissues. Biotin labeled probes containing specific response elements for PPARγ (PPRE) or LXR (LXRE) were incubated with nuclear protein extracts and separated by a 6% polyacrylamide gel. Representative blots and quantification for (**A**) PPARγ and (**B**) LXR were shown. For both blots, lane 1: free probe control; lane 2: nuclear protein control; lane 3 and 5: DNA-protein complex for samples from CON; lane 4 and 6: DNA-protein complex for samples from CLA; lane 7-9: competition with non-labeled probes at increasing concentrations. Shifted bands were quantified and compared between CON and CLA. Difference was considered significant when $P < 0.05$.

Chapter 6

Conclusions

The biosynthesis of milk fat relies on many lipogenic enzymes catalyzing the reactions in each step of the synthetic pathways. As proteins, lipogenic enzymes can be regulated at the transcriptional level where RNA is transcribed from DNA. During this process, transcription factors play very important roles in controlling the transcription of genes. Specific to lipid synthesis, SREBP-1, PPARγ and LXR have been identified as key transcription factors contributing to the regulation of lipogenic genes. Using specific SREBP-1 siRNA to knock down SREBP-1 revealed that SREBP-1 broadly regulates lipogenic genes involved in *de novo* FA synthesis, preformed FA uptake, FA desaturation and trafficking, and TAG synthesis in bovine mammary epithelial cells. The results from this *in vitro* experiment indicated a central regulatory role for SREBP-1 in lipid synthesis. Likewise, the positive effects of PPARγ and LXR activation on lipid synthesis and lipogenic gene expression in bovine mammary epithelial cells have been determined using their agonists (Kadegowda et al., 2009; McFadden and Corl, 2010). It is highly possible that PPARγ and LXR regulate lipogenic gene transcription during lipid synthesis in bovine mammary epithelial cells. When milk fat synthesis is influenced by the environment, the underlying mechanism is altered expression or activity of these lipogenic enzymes and therefore the transcription factors become potential regulatory points under these conditions. Further studies are needed to confirm the interactions between transcription factors and promoters of lipogenic enzyme genes.

Understanding the regulation of milk fat synthesis by a transcription factor can be complicated by self-regulation or cross talk with other factors. Although the crosstalk between

transcription factors was not directly examined in these studies, efforts to establish a network for SREBP-1, PPARγ, and LXR have been made. Promoter analysis has revealed a cluster of binding sites (SRE complex), composed of an NFY site, a SRE and an Sp1 site, in the promoter of mouse SREBP-1c, indicating regulation of SREBP-1c by itself through a feed-forward loop (Amemiya-Kudo et al., 2000). The linkage between SREBP-1 and LXR has been well established by studies characterizing an LXRE in the bovine SREBP-1c promoter and increased SREBP-1 expression by LXR activation in bovine mammary epithelial cells (Lengi and Corl, 2010; McFadden and Corl, 2010).

To draw a more complete network for the three transcription factors and determine whether there are connections between PPARγ and LXR or between PPARγ and SREBP-1, additional research will be required. There is a lack of studies on these relationships in bovine mammary tissue, but reports from experiments using other types of cells and tissues illustrate possibilities in the bovine mammary gland. In 3T3-L1 cells, SREBP-1 activates PPARγ promoters and directly induces PPARγ transcription (Fajas et al., 1999). Additionally, SREBP-1 promotes production and secretion of lipids in 3T3-L1 cells that are ligands for PPARγ, and therefore the lipids further increase PPARγ activity (Kim et al., 1998b). In macrophages, LXRα is known to be a target of and regulated by PPARγ (Chawla et al., 2001). Clarification of regulatory networks in bovine mammary epithelial cells could provide a possibility for strategies to more efficiently influence milk fat production.

As an important example of milk fat influenced by the environment, diet-induced MFD has been studied for a long time. After *trans*-10, *cis*-12 CLA was identified as a FA causing MFD, the question of how this FA affects milk fat synthesis has been raised. As possible regulators of milk fat synthesis, the roles of SREBP-1, PPARγ and LXR in MFD need to be

determined. We confirmed that SREBP-1 activation is likely a control point of milk fat synthesis during MFD from our study using luciferase reporters to evaluate its activation under *trans*-10, *cis*-12 CLA treatment. Previous studies have also shown a reduction in SREBP-1 expression in MFD that may be due to self-regulation of SREBP-1 (Harvatine and Bauman, 2006). Our results show an inhibitory effect of *trans*-10, *cis*-12 CLA on SREBP-1 activation more directly and are consistent with the observations of reduced proteolytic activation of SREBP-1 by *trans*-10, *cis*-12 CLA in MAC-T cells (Peterson et al., 2004). Contrary to SREBP-1, PPARγ and LXR were not affected by *trans*-10, *cis*-12 CLA directly in MAC-T cells. Whether they are regulated by *trans*-10, *cis*-12 CLA *in vivo* needs to be determined by further studies. The distinct responses of SREBP-1 and LXR activation to *trans*-10, *cis*-12 CLA offer little support for the hypothesis of LXR-mediated SREBP-1 regulation during inhibition.

The physiological and biological processes between a cell culture system and an organism can vary largely. Therefore, it is essential to verify our *in vitro* observations under *in vivo* conditions. Transcription factor activation in bovine mammary gland was examined in our study and investigation of the effect of *trans*-10, *cis*-12 CLA on transcription factor expression and activation in the mammary gland was attempted, but results were largely inconclusive. More *in vivo* studies are required to reveal the interactions between transcription factors and lipogenic genes and to determine the role of these interactions, if any, in milk fat regulation. With knowledge of transcription factor-mediated milk fat synthesis, crosstalk between transcription factors, and *trans*-10, *cis*-12 CLA affecting milk fat through transcription factors, strategies targeting the regulatory factors can be made to control milk fat production.

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Appendix A

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