ABSTRACT

Title of document: DENOVO SYNTHESIZED FATTY ACIDS AS REGULATORS OF MILK FAT SYNTHESIS

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The objectives of the dissertation research were to determine the role of *denovo* synthesized fatty acids (DNFA) in the regulation of milk fat synthesis. Milk fat responses to increasing amounts of short- and medium-chain fatty acids (SMCFA), added in the proportion as synthesized *denovo,* were studied in lactating dairy cows. The results showed a significant linear increase in milk fat concentration with SMCFA supplementation. However, milk fat yield was similar for all treatments.

A subsequent study was aimed at increasing the availability of SMCFA during *trans*-10, *cis*-12 CLA-induced milk fat depression (MFD) in lactating dairy cows to determine whether SMCFA can rescue part of CLA-induced MFD. Post-ruminal infusion of butterfat (BF) was used as a source of SMCFA. The BF treatment was compared to a mixture of fats containing only the long-chain FA (LCFA) with or without *trans*-10, *cis*-12 CLA infusion. Milk fat content and yield were significantly reduced with *trans*-10, *cis*-12 CLA. However, increased availability of SMCFA with BF infusion had no effects on milk fat yield and concentration. *Trans*-10, *cis*-12 CLA significantly

reduced the mRNA expression of transcription factor *SREBP-1c* along with its downstream targets including *ACC, FASN, LPL, SCD* and *AGPAT*. The increased availability of SMCFA had no effect on either lipogenic gene or protein expression suggesting that nutritional manipulation was not sufficient to rescue *trans*-10, *cis*-12 CLA-induced MFD.

Finally, the effects of combination of a Rosiglitazone (ROSI), a *PPAR-γ* agonist, and *trans*-10, *cis*-12 CLA were examined on mammary and hepatic lipogenesis in lactating mice. Mammary lipogenesis was significantly reduced with *trans*-10, *cis*-12 CLA, reducing the milk fat content and mRNA expression of lipogenic transcription factors *SREBP1-c* and *PPAR- γ. Trans*-10, *cis*-12 CLA significantly increased hepatic lipid accumulation, while the mRNA expression of *SREBP1-c* and *PPAR- γ* were not altered. On the contrary, ROSI had no effects on mammary lipogenesis. However, ROSI significantly rescued *trans*-10, *cis*-12 CLA-induced hepatic steatosis.

DENOVO SYNTHESIZED FATTY ACIDS AS REGULATORS OF MILK FAT SYNTHESIS

By

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Dedication

…….. to my family

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List of abbreviations

ABC	ATP binding cassette
ACBP	Acyl-CoA binding protein
ACC	Acetyl-CoA carboxylase
ACO	Acyl-CoA oxidase
AGPAT	Acylglycerol-3-phosphate acyltransferase
BH	Biohydrogenation
ChREBP	Carbohydrate response element binding protein
CO	Coconut oil
CPT-1	Carnitine palmitoyl transferase
CD36	Clusters of differentiation-36
CLA	Conjugated linoleic acid
DG	Diglyceride
DHA	Docosahexaenoic acid
DNFA	*Denovo* synthesized fatty acid
DGAT	Diacylglycerol acyltransferase
DMI	Dry matter intake
EPA	Eicosapentaenoic acid
FABE	Fatty acid butyl ester
FABP	Fatty acid binding protein
FAME	Fatty acid methyl ester
FASN	Fatty acid synthase
FAT	Fatty acid transporter
FCM	Fat corrected milk
FFA	Free fatty acids
G6P	Glucose 6-phosphatase
GPAT	Glycerol-3-phosphate acyltransferase
INSIG-1	Insulin induced gene
IL-6	Interleukin-6
LPL	Lipoprotein lipase
LCFA	Long-chain fatty acid
LC-PUFA	Long-chain polyunsaturated fatty acid

MG	Monoglyceride
MCFA	Medium-chain fatty acid
MCP	Multiple component pricing system
MCT	Medium-chain triglyceride
MCT-1	Monocarboxylate transporter-1
MFD	Milk fat depression
MUFA	Monounsaturated fatty acid
NE_L	Net energy of lactation
NEFA	Non-esterified fatty acid
NAFLD	Non-alcoholic fatty liver disease
PEPCK	Phospho-enol pyruvate carboxykinase
PGE_2	Prostaglandin E2
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	Peroxisome proliferator activated receptor-γ
PUFA	Polyunsaturated fatty acid
S1P	Site1 protease
S2P	Site2 protease
SCAP	SREBP cleavage activating protein
SCC	Somatic cell count
SCD	Stearoyl-CoA desaturase
SCFA	Short-chain fatty acid
SREBP	Sterol regulatory element binding protein
SMCFA	Short-and medium-chain fatty acid
SRE	Sterol response element
TG	Triglyceride
TNF-α	Tumor necrosis factor-α
VFA	Volatile fatty acid
VLDL	Very low density lipoprotein
RXR	Retinoid X receptor

Chapter 1: INTRODUCTION

The composition of milk has been an important determinant of profitability for the dairy industry ever since the USDA Federal Milk Marketing System adopted a multiple component pricing system (MCP). This has changed the milk payment system for dairy producers to one based on the amounts of milk components produced rather than milk volume. Milk fat and milk protein are the most valuable milk components as compared with other solids (lactose and minerals) demonstrating powerful economic incentives for dairy producers to produce greater amounts of milk fat and milk protein (USDA-NASS, 2009).

Dietary manipulations in lactating cows offer a means for making rapid changes in the milk composition (Sutton, 1989). Milk fat responses to dietary manipulations have much greater impact on milk fat concentration and yield than on milk protein or other milk solids (lactose and minerals)(Sutton, 1989) . This signifies the importance of studying the metabolic regulation of milk fat synthesis. This would further help in developing practical guidelines for dairy farmers to better control the quality and quantity of milk fat according to market demands.

Milk fat synthesis depends on two general sources of fatty acids (FA); i.e. *denovo* FA synthesis in mammary gland and transfer of preformed FA from blood triglycerides (TG). Short-and medium-chain FA (SMCFA) including FA from C4-C14 and half of C16 are synthesized *denovo* while long-chain FA (LCFA) either originate from diet or are mobilized from adipose TG as preformed FA (Moore and Steele, 1968).

Milk fat content and yield can be reduced up to 50% (deVeth et al., 2003) by diets containing high levels of concentrates and polyunsaturated fatty acids (PUFA). The low milk fat syndrome commonly termed as milk fat depression (MFD) has been linked to unique FA intermediates produced during biohydrogenation (BH) of unsaturated FA in rumen (Bauman and Griinari, 2003). It has been shown that when intermediates in the BH process such as *trans*-10, *cis*-12 CLA accumulate, they are absorbed in the small intestine and subsequently interfere with milk fat synthesis in the mammary gland, resulting in MFD (Baumgard et al., 2000).

Diet-induced MFD can also provide insights on regulatory aspects of milk fat synthesis. Milk fat depression is characterized by reduced mRNA abundance and enzyme activity of several mammary lipogenic enzymes in lactating dairy cows (Piperova et al., 2000; Gervais et al., 2009), mice (Lin et al., 2004; Kadegowda et al, 2010) and rats (Ringesis et al., 2004). The coordinated downregulation of mammary lipogenic gene expression suggests transcriptional regulation of mammary lipogenesis (Harvatine and Bauman, 2006). *Sterol regulatory element binding protein -1c* (*SREBP-1c)* has been implicated as a major transcriptional regulator of mammary lipogenesis (Harvatine and Bauman, 2006). The mRNA abundance of *SREBP-1c* is downregulated by *trans*-10, *cis*-12 CLA in mice (Kadegowda et al., 2010), lactating dairy cows (Harvatine and Bauman, 2006) and in bovine mammary epithelial cells (Peterson et al., 2004). However, *SREBP-1c* knockout mice failed to exhibit complete suppression of mammary lipogenesis, suggesting the role of other transcription regulators in FA synthesis (Liang et al., 2002). Recently, Bionaz and Loor (2008) proposed a pivotal role of *PPAR-* γ in controlling milk fat synthesis by serving as a regulator for *SREBP* activity. Further, a recent study

(Kadegowda et al., 2009) also showed marked upregulation of mammary lipogenic gene expression with Rosiglitazone, a *PPAR-γ* agonist, in bovine mammary epithelial cells. However, in their study *trans*-10, *cis*-12 CLA had no effects on *PPAR-γ* gene expression.

The milk FA profile during *trans*-10, *cis*-12 CLA-induced MFD is characterized by reduced secretion of FA of all chain lengths. However, the effects are more pronounced for SMCFA than LCFA, suggesting inhibition of *denovo* FA synthesis (Bauman and Griinari, 2003). Short-and medium-chain FA are important for milk TG synthesis (Moore and Christie, 1979) and for maintaining fluidity of milk fat (Barbano and Sherbon, 1980). Since SMCFA accounts for approximately 50% of FA in ruminant's milk (Moore and Christie, 1979) and 20-50% in rodent's milk (Grigor MR, 1984) and are not present in typical feedstuffs, the only source for SMCFA is *denovo* FA synthesis in mammary gland. Recently, the importance of SMCFA during milk fat synthesis was underscored when post-ruminal infusion of butterfat, used as a source of SMCFA, increased milk fat synthesis in lactating dairy cows (Kadegowda et al., 2008). This suggests that the mammary gland is responsive to SMCFA during normal milk fat synthesis. However, one could hypothesize that the responsiveness to SMCFA might be greater during MFD when the synthesis of these FA is inhibited.

Thus, the basic premise of this study is that *denovo* synthesized FA (DNFA) are limiting for milk fat synthesis and providing supplemental DNFA might prevent reduction in the milk fat yield and content during MFD. The central hypothesis of this proposal is that "***the availability of denovo synthesized FA is the key limiting substrate for milk fat synthesis***".

Chapter 2: LITERATURE REVIEW

Milk fat is the main energy component in milk and is also responsible for many of the physical, organoleptic and manufacturing properties of dairy products (Jensen, 2002). Milk fat is mainly composed of triglycerides (TG) with small amounts of phospholipids, cholesterol, diglycerides (DG), monoglycerides (MG) and free FA (FFA) (Jensen, 2002) and its synthesis is highly responsive to nutrition. Nutritional manipulation of milk fat provides a practical tool to alter its yield and composition (Bauman and Griinari, 2003). To appreciate fully the effect of nutrition on milk fat composition, it is important to be familiar with the metabolic pathways responsible for milk fat synthesis.

Milk fat synthesis

The basic mechanism of milk fat synthesis depends on two general sources of FA; i.e. *denovo* synthesis of FA in mammary gland and uptake of preformed FA from blood TG. The short-and medium-chain FA (C4-C14) and approximately 50% of C16 are synthesized within the mammary gland, whereas the remaining 50% of C16 and other long-chain FA (LCFA) are derived from the blood TG in circulating chylomicra and very low density lipoproteins (VLDL) (Bauman and Davis, 1974).

Denovo fatty acid synthesis

Denovo synthesis of FA is achieved by a sequence of reactions involving condensation of two-carbon (C-2) units derived initially from acetyl-CoA (Smith, 1994). Glucose, FA and glycerol are utilized as substrates for FA synthesis. However in ruminants the substrates for milk fat biosynthesis come from the volatile FA (VFA); acetate and β hydroxy butyrate; produced during rumen fermentation. *Denovo* FA

synthesis is catalyzed by two key enzymes – *Acetyl-CoA carboxylase (ACC) and Fatty acid synthase (FASN).* Both enzymes are abundantly expressed in lipogenic tissues including liver, adipose tissue and lactating mammary gland (Kim, 1997).

Acetyl-CoA carboxylase is a biotin containing enzyme catalyzing conversion of acetyl-CoA and biocarbonate to malonyl-CoA, a first rate limiting reaction in milk FA synthesis (AbuElheiga et al., 1997). *ACC* is responsible for regulating the amounts of FA in cell (Kim, 1997) with its two isozymes; *ACC-α* and *ACC-β*; involved in FA synthesis and oxidation, respectively (Wakil and Abu-Elheiga, 2009). While *ACC-α* is expressed more in lipogenic tissues and provides malonyl-CoA to the cells for FA synthesis (Lopezcasillas et al., 1991), *ACC- β* is expressed more in heart and skeletal muscles (AbuElheiga et al., 1997) and controls the mitochondrial FA oxidation. The role of *ACC-β* is supported by the fact that *carnitine palmitoyl transferase (CPT-1),* an essential component for mitochondrial oxidation, is extremely sensitive to inhibition by malonyl CoA generated by *ACC- β* (Kim, 1997). The activity of *ACC* can be regulated at various levels including short-term control with allosteric modulation by different metabolites (citrate, glutamate, free fatty acids, malonyl CoA, etc.) and reversible phosphorylation (AMP kinase, c-AMP dependent protein kinase, ACC kinase, etc.) at the serine residues and long-term regulation involving hormonal and nutritional control of gene regulation (Kim, 1997, Mao et al., 2003).

Fatty acid synthase is a single multifunctional protein containing seven catalytic domains arranged in a series of connected globular domains (Wakil, 1989). These domains catalyze the elongation of acetyl-CoA by C-2 units derived from malonyl-CoA in a stepwise and sequential manner (Wakil, 1989). Six cycles of condensation result in

the formation of palmitic acid (C16), the predominant end product in eukaryotic cells (Bernard et al., 2008).

Acyl moieties greater than C16 cannot be elongated by *FASN* and are susceptible to hydrolysis by *thioesterase-I* liberating palmitic acid (C16). A second thioesterase, *Thioesterase II*, in some species (rodents, rabbit, and humans) overrides *thioesterase I* and results in early termination of FA synthesis, generating medium-chain FA (Barber et al., 1997). The presence of *thioesterase II* explains the varying proportions of medium-chain FA in different species. On the contrary, ruminants lack *thioesterase II*. However, short-and medium-chain FA (SMCFA) are synthesized in ruminants due to intrinsic thioesterase activity of *FASN* (Bernard et al., 2008). Ruminant *FASN* exhibits an intrinsic transacylase capable of loading and releasing acyl chain from two to twelve carbons in length (Knudsen and Grunnet, 1982).

Uptake of preformed FA by mammary gland

The incorporation of FA from plasma TG into milk fat involves their complete or partial hydrolysis by *lipoprotein lipase (LPL). LPL* is a member of the TG lipase family of proteins that exhibit significant TG esterase activity (Wang and Eckel, 2009). This enzyme is highly expressed in the lactating mammary gland and originates from mammary adipocytes unlike in other tissues such as skeletal muscle and adipose where it is synthesized in parenchymal cells and spreads along the vascular mesh (Wang and Eckel, 2009). The mammary activity of LPL markedly increases immediately prior to parturition and remains elevated throughout lactation, accompanied by concomitant downregulation in adipose tissue (Shirley et al., 1973). The site of its activity in the

lactating mammary gland is the capillary lumen where it captures and hydrolyzes TG-rich lipoproteins to release FA (Neville and Picciano, 1997).

The FA released from TG and circulating non-esterified FA (NEFA) crosses the capillary endothelium and interstitial space to reach the mammary epithelial cell. Further, FA crosses the plasma membrane either by diffusion or a saturable transport system (Bernard et al., 2008). Several FA transporters including *acyl-CoA binding proteins* (*ACBP)*(Knudsen et al., 2000), *clusters of differentiation (CD36)*(Abumrad et al., 2000), *fatty acid binding proteins (FABP)* (Lehner and Kuksis, 1996) and *ATP binding cassette (ABC) transporters* (Klein et al., 1999) have been suggested as playing important roles in regulating FA transport and FA concentration in the cytoplasm of mammary epithelial cells.

Mammary epithelial cells contain an active *stearoyl-CoA desaturase* (*SCD)* enzyme. The mRNA abundance and enzyme activity of *SCD* increases at the onset of lactation, suggesting its importance during milk fat synthesis (Kinsella, 1970, Ward et al., 1998). *SCD* catalyzes the δ-9 desaturation of FA substrates by introducing a *cis* -9 double bond mainly in C14 to C19 FA (Bernard et al., 2008), converting saturated FA to monounsaturated FA (Grummer, 1991). About 40% of stearic acid taken up by the gland is desaturated, contributing to more than 50% of oleic acid secreted in milk fat (Bickerst et al., 1974, Chilliard et al., 2000). In bovine mammary gland, *SCD* is also responsible for synthesis of the major part of *cis*-9, *trans*-11 CLA isomer (Corl et al., 2001).

Triglyceride (TG) synthesis

The synthesis of TG involves addition of FA to the *sn*-1, *sn*-2, and *sn*-3 positions of the glycerol backbone (Bernard et al., 2008). The major pathway for TG biosynthesis in the mammary gland is the *sn*-glycerol 3-phosphate pathway involving the formation of phosphatidic acid using two acyl moieties (Dils, 1983). The first step in TG biosynthesis is catalyzed by *glycerol-3-phosphate acyl transferase (GPAT)* where FA are esterified with glycerol-3-phosphate at the *sn*-1 position. The second step is committed by *acyl glycerol phosphate acyl transferase (AGPAT)* which catalyzes FA esterification at the *sn*-2 position. *Diglycerides (DG)* is synthesized by hydrolyzing the phosphate group at *sn*-3 position with enzyme phosphatidate phosphatase (Moore and Christie, 1979).The final step of TG synthesis is catalyzed by *di-acyl glycerol acyl transferase (DGAT). DGAT* is the only protein that is specific to TG synthesis and therefore might play an important regulatory role (Mayorek et al., 1989).

The position of FA along the glycerol backbone affects the nutritional and functional attributes of milk fat (Bernard et al., 2008). Fatty acids are not distributed randomly on *sn*-1, *sn*-2, and *sn*-3 positions. In ruminants, short chain FA, SCFA (C4-C6) are almost exclusively (95%) esterified to the *sn*-3 position in milk fat. All of the C4, 93% of C6 and 63% of the C8 have been shown to be esterified to the *sn*-3 position (Jensen, 2002). The distributions of other FA in the TG appear to vary depending on the molecular weight of the TG (Parodi, 1982). The C10, C12 and C14 FA are predominantly found in the *sn*-2 position. Palmitate (C16:0) is almost equally distributed between the *sn*-1 and *sn*-2 positions. Oleate (C18:1) is preferentially distributed in the *sn*-3 position in high molecular weight TG and at *sn*-1, in the low molecular weight TG whereas stearate

(C18:0) selectively esterifies at the *sn*-1 position (Parodi, 1982). Unsaturated FA are preferentially esterified at the *sn*-3 position in higher molecular weight TG. Though the exact mechanisms involved in the positioning of the FA in milk fat TG in ruminants is not clearly understood, specific mammary acyltransferases may be involved (Parodi, 1982).

Transcriptional regulation of lipid synthesis

Based on previous studies it has been suggested that genes involved in milk fat synthesis might share a common regulatory mechanism via transcription factors (Clarke, 2001). The major transcription factors involved in lipid metabolism are sterol regulatory element binding protein-1 and *Peroxisome proliferator activated receptors (PPARs)* (Bernard et al., 2008).

Sterol regulatory element binding protein (SREBP)

Sterol regulatory element binding protein is the major transcriptional factor associated with regulation of cholesterol and lipid metabolism (Brown and Goldstein, 1997). *SREBP* are members of the basic-helix-loop-helix-leucine zipper family of transcription factors synthesized as a 1150 amino acid precursor that is attached to the nuclear envelope and membrane of the endoplasmic reticulum (Wang et al., 1994). These are structurally composed of 3 segments with two membrane spanning regions. The –NH_2 terminal transcription factor domain consists of ~480 amino acids, –COOH regulatory domain consists of ~590 amino acids and one hydrophobic region of ~80 amino acids contains two hydrophobic transmembrane segments (Brown and Goldstein, 1997)

Following translation, *SREBP* precursors bind to the *SREBP cleavage activating protein (SCAP)* (Sakai et al., 1998). *SCAP* interacts with *INSIG-1* (Insulin induced gene) proteins, which retain the *SCAP/SREBP* complex in the ER compartment (Yang et al., 2002). The *SREBP* cleavage can be controlled by cellular sterol content due to the presence of a sterol sensing domain on *SCAP* (Nohturfft et al., 1998). The *SREBP-SCAP* complex is retained on the ER in the presence of high sterol concentrations, while during low sterol concentrations ,the *SREBP-SCAP* complex is detached from INSIG proteins (Figure 2.1), allowing the *SCAP* to escort *SREBP* to the golgi apparatus (Sakai et al., 1998). Upon activation, the *SREBP* precursor undergoes a sequential two-step cleavage process by two proteases, site1 protease (S1P) and site 2 protease (S2P) to release a 68-kDa mature *SREBP* (Sakai et al., 1998, Wang et al., 1994), then the mature protein is translocated to the nucleus where it binds the target genes on sterol response elements (SRE) as a homodimer.

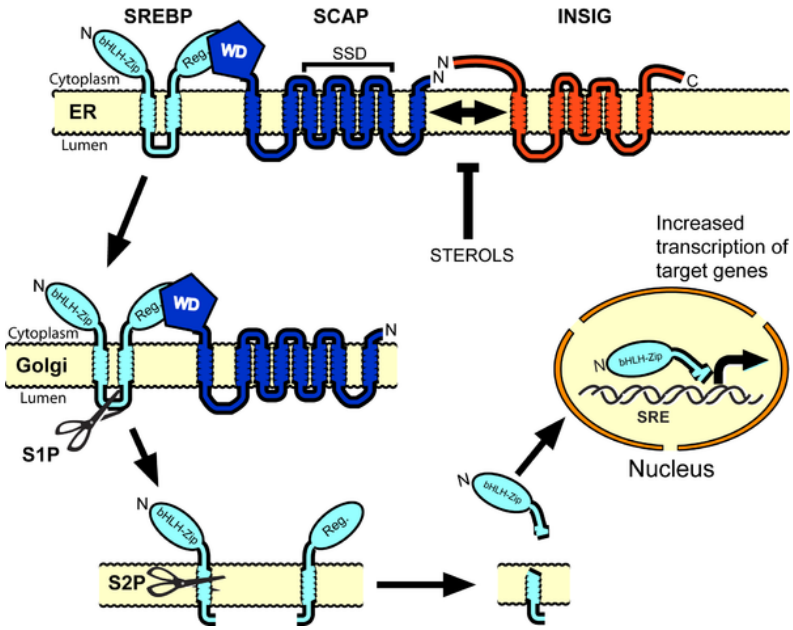

Figure 2.1. The *SREBP* pathway (Brown and Goldstein, 1997)

There are three isoforms of *SREBP- 1a*, *1c* and *2*. Previous studies have demonstrated that *SREBP* isoforms function differently. *SREBP-1* is involved in regulation of FA metabolism and predominately regulates enzymes involved in fat synthesis. It is the major regulator of mammary lipogenesis and is expected to be the predominant lipid regulatory transcript expressed in mammary tissue (Harvatine and Bauman, 2006). In mice, *SREBP1* is upregulated at the initiation of lactation (Rudolph et al., 2007), and its disruption results in a 41% decrease in milk fat concentration. *SREBP-2* preferentially activates genes involved in the cholesterol pathway (Horton et al., 2003).

Peroxisome proliferator activated receptor- γ (PPAR-γ)

Peroxisome proliferator activated receptors are the sub-family of nuclear receptors that regulate lipid metabolism and adipocyte differentiation. Three isotypes of PPAR have been identified to date namely PPAR-α, PPAR-β and PPAR-γ. All three

isotypes are generally co-expressed in all cell types but their relative levels of expression vary from one cell to another (Jain et al., 1998). PPAR heterodimerize with *retinoid X receptor (RXR)* and regulate the expression of genes containing the peroxisome proliferative response element (PPRE) (Berger and Moller, 2002).

Peroxisome proliferator activated receptors-*γ* is an important member of the nuclear receptor super family of transcription factors that can be activated by lipophilic ligands. It regulates adipocyte differentiation and has been implicated as a key protein for thermogenesis and lipid metabolism in adipose tissue (Jain et al., 1998). It also suppresses macrophage cytokine production reducing inflammatory responses (Jiang et al., 1998). While *PPAR-γ* is known to promote adipogenesis in adipose, its role in the mammary gland is still uncertain (Wan et al., 2007). Targeted deletion of *PPAR-γ* gene in mice resulted in production of milk containing elevated levels of inflammatory lipids causing alopecia, inflammation, and growth retardation in mouse pups following milk consumption (Want et al., 2007). This suggests a pivotal role of *PPAR-γ* in maintaining milk quality and suppressing the production of inflammatory lipids. Bionaz and Loor (2008) showed increased expression of *PPAR-γ* with the onset of lactation and proposed that the role of *PPAR-γ* in lactating mammary gland might be pivotal by controlling the activities of *INSIG-1* which further controls the formation of active *SREBP-1*. Simultaneous increases in genes related to FA uptake, transport synthesis, and desaturation were also observed, suggesting that the part of LCFA effects may have been mediated through *PPAR-γ* (Bionaz and Loor, 2008). Recently, Kadegowda et al., (2009) showed that the activation of *PPAR-γ* via its agonist Rosiglitazone in bovine mammary epithelial cells (MAC-T) upregulates mammary lipogenic mRNA expression including

gene expression for *denovo* FA synthesis. *PPAR-γ* also activates a subset of transcription factors namely *SREBP-1*and *2*.

Nutritional regulation of milk fat synthesis

Various factors influence milk fat synthesis in dairy cattle including genetics, breed, stage of lactation, environment, parity and nutrition. This section deals only with nutritional regulation of milk fat synthesis. The nutritional control of milk fat synthesis has been extensively studied to improve the manufacturing properties of milk and to enhance the beneficial fatty acids in milk fat (Jenkins and McGuire, 2006). Various factors like amount and type of roughage, forage particle size, roughage to concentrate ratio, amount and type of lipids, intake and meal frequency are among the most important dietary factors affecting milk fat synthesis, and subsequently, FA composition (Sutton, 1989).

Milk FA composition is markedly affected by the FA composition of the diet in most species (Neville and Picciano, 1997). However, in ruminants, the FA profile of the diet is markedly altered by ruminal microbial metabolism; thus, milk FA composition does not reflect the dietary FA profile. However diet can still have major effects on milk fat synthesis even in ruminants as it can markedly affect the microbial population and rumen microbial processes (Bauman and Griinari, 2003).

Rumen metabolism

When dietary constituents enter the rumen, a wide range of chemical transformations occur as a result of microbial fermentation (Harfoot, 1978). Dietary lipids are hydrolyzed to free fatty acids by microbial lipases and unsaturated free FA are further biohydrogenated into saturated FA as a detoxification mechanism to protect rumen microbes (Henderso, C. 1973). This results in marked differences between the FA profile of the diet and FA profile of the lipids leaving the rumen (Harfoot and Hazlewood, 1988).

The earliest evidence of ruminal biohydrogenation (BH) was observed when linolenic acid (C18:3) content of linseed oil was significantly reduced in ruminal fluid of sheep accompanied by a concomitant increase in the content of linoleic acid (C18:2) (Reiser, 1951, Shorland et al., 1955). Several studies have shown that the first step in BH of both linolenic and linoleic acid is isomerization of the *cis*-12 bond, forming several monoene and diene derivatives containing *trans*-11 bonds (Figure 2.2) (Harfoot and Hazlewood, 1988). Recently the presence of multiple CLA intermediates (*cis*-10, *cis*-12, *cis*-9, *cis*-11 and *trans*-8, *trans*-10) during linolenic and linoleic acid BH have indicated that the pathways are much more complex than initially reported and include several other intermediates before eventual conversion to stearic acid (Jenkins et al., 2008, Lee and Jenkins, 2011). The conversion of oleic acid to stearic acid involves formation of various *trans*-C18:1 intermediates with double bonds at 6,7,9-16 positions (Mosley et al., 2002). The rumen BH also occurs on C20 and C22 FA. The disappearance of C20:5 (eicosapentanoic acid, EPA) and C22:6 (docosahexaenoic acid, DHA) is extensive however these FA do not become fully saturated. Instead numerous intermediate

compounds are produced (Chilliard et al., 2000). Due to an unknown mechanism the addition of DHA and/or EPA increases the ruminal production of *trans* C18:1 FA. Possibly, DHA and EPA are either converted to *trans* C18:1 isomers or increases the formation of *trans* 18:1 isomers from C18 unsaturated FA biohydrogenation. Recent study, using uniformly ^{13}-C-labeled DHA, has suggested that supplementing DHA alters the rumen BH pathways resulting in increased *trans* FA isomers that are absorbed and transferred into milk (Klein and Jenkins, 2011).

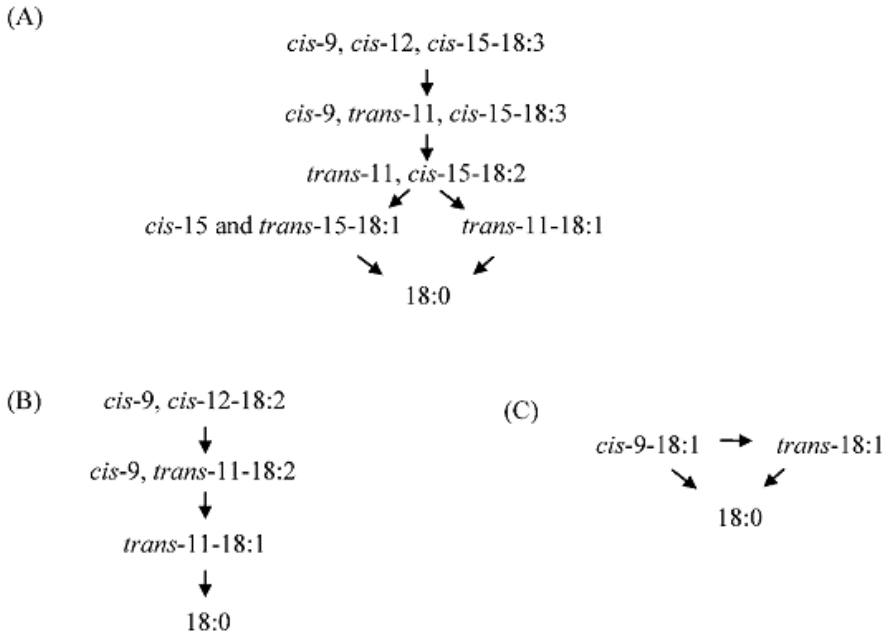

Figure 2.2. Biohydrogenation pathways of unsaturated FA (Harfoot and Hazlewood, 1988)

The extent of BH is dependent on the type of diet (Chilliard et al., 2000). High concentrates can reduce the pH in rumen and shift the profile of the microbial population and volatile FA (VFA) pattern. It further limits lipolysis, and subsequently BH, which occurs only on free FA (Chilliard et al., 2000). In the case of a marine oil diet, the rumen pH and VFA pattern are not affected. However, the FA in marine oil alter microbial processes by directly affecting critical steps in BH processes, further altering the end

products of ruminal BH. As a result of diet-induced alternations in rumen fermentation, unique FA intermediates including *trans*-10 C18:1 and CLA isomers accumulate which are potent inhibitors of milk fat synthesis (Klein and Jenkins, 2011). In the following section the role of CLA isomers on mammary and hepatic lipid metabolism will be discussed.

Conjugated linoleic acid (CLA)

Conjugated linoleic acid refers to a group of dienoic derivatives of linoleic acid with conjugated double bonds arranged in different combinations of *cis* and *trans* configuration (Pariza et al., 2001). Currently, 16 naturally occurring CLA isomers have been identified with different positional (7/9, 8/10, 9/11, 10/12, 11/13) and geometric (*cis*/*cis*, *trans*/*trans*, *cis*/*trans*, *trans*/*cis*) combinations (Eulitz et al., 1999; Sehat et al., 1999).

The sources of CLA include those naturally present in dairy products and meat from ruminant animals or those contained in industrially hydrogenated vegetable oils such as margarines and other synthetic products (Park and Pariza, 2007). The predominant CLA isomer originating from the ruminant products is *cis*-9, *trans*-11 CLA (>80%), with small amounts of *trans*-10, *cis*-12 CLA and other isomers (Parodi, 1977). The industrially synthesized CLA and other commercial products intended for human consumption typically consist of equal amounts of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA and other isomers (Chin et al., 1992). Of all of the CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA have been the most widely studied due to their biologically active properties (Pariza et al., 2001).

Physiological effects of CLA

Much of the current interest in dietary CLA is due to their anti-carcinogenic (Chin et al., 1992), anti-atherogenic (Lee et al., 1994), and immunity enhancing properties (Miller et al., 1994), and effects on body composition (Park et al., 1997). Each CLA isomer has unique bioactive properties, and hence, the biological effect from a mixture of dietary CLA isomers, as is the case in most of the studies, would be the combined effect of their distinct isomers (Pariza et al., 2001). For example, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA have additive effects on cancer (Ip et al., 2002), and immune cell functions (Belury, 2002) but are antagonistic with respect to insulin sensitivity (Park and Pariza, 2007). While *cis*-9, *trans*-11 CLA improves insulin sensitivity, *trans*-10, *cis*-12 CLA causes insulin resistance (Park and Pariza, 2007). Also, *trans*-10, *cis*-12 CLA is solely responsible for changes in body composition and reducing adipose mass (Park et al., 1999b).

Body Weight and Lean Mass

CLA reduces body weight and body fat mass and increases lean mass in different species (Park et al., 1997). However, the response appears to vary depending on species, physiological stage, and fat depot (Larsen et al., 2003, Park et al., 1997). Table 2.1 provides a summary of studies reviewed across species with respect to body weight and adiposity where the number of experiments showing significant ($P < 0.05$) increases, decreases, or no change, and the mean response to dietary *trans*-10, *cis*-12 CLA within those categories are reported. The range of *trans*-10, *cis*-12 CLA addition in these studies varied between 0.1 to 1% of the diet.

Trans-10, *cis*-12 CLA reduces body fat to a maximum extent in mice (60 to 80%) (Ide, 2005; Andreoli et al., 2009). However, modest and inconsistent effects are seen in rats (Purushotham et al., 2007; Gudbrandsen et al., 2009) hamsters (9 to 58%) (Zabala et al., 2006; Miranda et al., 2009) and pigs (6 to 25%) (Whigham et al., 2007). Similarly, variable responsiveness to CLA was observed for epididymal, perirenal and subcutaneous body fat depots (Zabala et al., 2006). Inconsistent responses to *trans*-10, *cis*-12 CLA have been reported in clinical trials with humans (Bhattacharya et al., 2006). Some have shown significant effects on body composition (Blankson et al., 2000, Thom et al., 2001) while others have not (Zambell et al., 2000; Petridou et al., 2003). The differences in the responses are attributed to differences in the dose levels, age, and rate of adipose tissue TG turnover (Malpuech-Brugere et al., 2004; Bhattacharya et al., 2006; Park and Pariza, 2007). The response to CLA isomers also depends on the physiological state of the animal, which is probably due to differences in the preferential uptake of CLA by different tissues. For example, *trans*-10, *cis*-12 CLA is preferentially taken up by the mammary tissue during lactation leading to a substantial (~45%) decrease in milk lipid synthesis (Kadegowda et al., 2010).

Effects of CLA on hepatic lipid metabolism

Liver plays an important role in energy homeostasis as it converts excessive dietary glucose into FA, which are exported as TG. Liver is an important target tissue for CLA effects irrespective of the physiological condition**.** Of the different CLA isomers, *trans*-10, *cis*-12 CLA causes increased lipid accumulation leading to hepatic steatosis (Clement et al., 2002; Degrace et al., 2003; Rasooly et al., 2007; Cooper et al., 2008). However, the intensity of lipid accumulation varies depending on the CLA concentration

in the diet, duration of feeding, physiological condition, and animal species (Table 2.1). The factors leading to hepatic lipid accumulation are multi-factorial involving increased FA influx, increased FA synthesis and altered FA oxidation and TG secretion insufficient to prevent lipid accumulation (Jourdan et al., 2009). These mechanisms are probably not mutually exclusive, and could act in a coordinated manner to hasten the development and progression of fatty liver (Gentile and Pagliassotti, 2008).

Hepatic FA Synthesis

Under normal conditions *denovo* lipogenesis contributes minimally to the lipid pool in the liver (Diraison and Beylot, 1998). However, the lipid synthesis increases to as much as 26% during steatotic conditions (Figure 2.3) (Donnelly et al., 2005). The increase in hepatic lipid content due to CLA, specifically *trans*-10, *cis*-12 CLA, is commonly associated with increased hepatic lipogenesis (Clement et al., 2002). In mice, CLA has been repeatedly shown to increase the expression of *SREBP-1c,* a key transcriptional regulator in hepatic lipogenesis and its down-stream genes *acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), and stearoyl-CoA desaturase-1 (SCD1)* (Clement et al., 2002; Takahashi et al., 2003; Liu et al., 2007) (Table 2.2). However, in rats and hamsters, the responses are equivocal. The increase in *SREBP-1c* expression in mice is attributed to hyperinsulinimia (Clement et al., 2002). The decreased expression of lipogenic *(ACC1, ACC2, FASN* and *SCD1*) genes in the absence of insulin in mice fed *trans*-10, *cis*-12 CLA further supports this argument (Jourdan et al., 2009). In addition to *SREBP-1c*, insulin induces the expression of *PPAR-γ* (Boelsterli and Bedoucha, 2002) which is in low abundance under normal conditions (Tontonoz et al., 1994). *PPAR-γ* expression is increased in steatotic liver (Clement et al., 2002, Zhang et al., 2006) while its ablation

ameliorates the condition in mice (Gavrilova et al., 2003). Insulin resistance in response to *trans*-10, *cis*-12 CLA could up-regulate genes of the glucogenic pathway (e.g., *PEPCK, G6P*) leading to hyperglycemia (Denechaud et al., 2007). In turn, elevated blood glucose concentrations could up-regulate hepatic lipogenesis through *carbohydrate response element binding protein* (*ChREBP),* a transcriptional regulator modulated by glucose. The targeted deletion of *ChREBP* in the liver improves the steatotic conditions in *ob/ob* mice (Denechaud et al., 2007). However, the role of *ChREBP* in CLA-induced hepatic steatosis is not known. Although hyperinsulinemia triggers the hepatic lipogenesis, CLA-induced hepatic steatosis in the absence of insulin suggests the involvement of other regulatory mechanisms affecting hepatic lipid accumulation (Jourdan et al., 2009).

Hepatic FA uptake and TG secretion

In experiments using mouse as experimental model, dietary *trans*-10, *cis*-12 CLA was associated with up-regulation of genes associated with FA uptake and TG secretion *(FAT/CD36*; Table 2.2, Figure 2.3). During hepatic steatosis 59% of hepatic TG is derived from free FA released from the adipose tissue and 15% is derived from dietary fat (Donnelly et al., 2005). FA transporters, (*FATP5, FAT/CD36, FABP-1, FABP-4, and FABP-5*) regulate the FA uptake by hepatocytes. While the over-expression of these proteins promotes steatosis, functional deletion ameliorates the condition (Doege et al., 2006; Zhou et al., 2008; Musso et al., 2009). As CLA are natural ligands and activators of *PPAR-γ* (Belury et al., 2002), the up-regulation of *FAT/CD36* by *trans*-10, *cis*-12 CLA (Degrace et al., 2006, Jourdan et al., 2009, Rasooly et al., 2007) could be through *PPAR-γ* leading to increased hepatic FA uptake. In addition to *FAT/CD36,* modest increases have been observed in the expression of *FABP-1* (1.39 fold) and *FABP-2* (1.7 fold) in liver of

lactating mice fed *trans*-10, *cis*-12 CLA (Kadegowda, A.K.G., Erdman, R.A., and Loor, J.J. Unpublished results).

Besides enhanced FA uptake and lipogenesis, alteration in very low density lipoprotein (VLDL) secretion rates could also result in hepatic fat accumulation (Nagayoshi et al., 1995). The VLDL production and secretion are increased in response to elevated lipid concentrations. However, impaired or insufficient fat export via VLDL predisposes animals to hepatic steatosis (Charlton et al., 2002). *Trans*-10, *cis*-12 CLA reduced TG secretion, leading to greater lipid accumulation in HepG2 cells due to reduced apolipoprotein B synthesis (Lin et al., 2001). Conversely, lipoprotein clearance was not affected in mice fed CLA (Degrace et al., 2003; Degrace et al., 2006). The TG export was increased with faster rates of VLDL secretion however it was insufficient to eliminate increased FA flux entering the liver, leading to hepatic steatosis (Degrace et al., 2003).

Hepatic FA Oxidation

Hepatic FA oxidation encompasses β-oxidation in mitochondria and peroxisomes and ω-oxidation in the microsomes (Reddy and Hashimoto, 2001). The FA from C8 to C20 are catabolized through the mitochondrial β-oxidation pathway while FA >C20 are initially catabolized in the peroxisomes to shorter FA which are then shuttled to mitochondria for further oxidation (Rasooly et al., 2007). Previous studies have reported variable responses in hepatic FA oxidation with *trans*-10, *cis*-12 CLA. Most of the studies have shown increased FA oxidation (Takahashi et al., 2003; Degrace et al., 2004; Javadi et al., 2004; Ide, 2005; Macarulla et al., 2005) while some have reported reduced (Rasooly et al., 2007) or unaltered FA oxidation (Park et al., 1997) with CLA.

Carnitine palmitoyltransferase-1 (CPT1) is the rate limiting enzyme for the mitochondrial β-oxidation pathway as it regulates the transport of fatty acyl CoA into mitochondria. When measured in mice, *CPT1* gene expression was consistently increased by CLA (Table 2.2) which might be mediated through transcriptional regulator *PPAR-α*, as it regulates the key enzymes (e.g., *CPT1, CPT2, ACO*) involved in hepatic FA oxidation (Moya-Camarena et al., 1999).

Despite increased FA oxidation, hepatic steatosis was consistently observed in mice fed CLA (Table 2.1, 2.2). Since studies showing increased FA oxidation were also associated with increased hepatic lipogenesis, it is possible that that the rates of hepatic lipogenesis far exceed the rates of FA oxidation, resulting in increased lipid accumulation. Along with increased lipogenesis, the level of malonyl CoA, a product of *ACC*, was also increased which allosterically inhibits *CPT1* enzyme activity (Degrace et al., 2004). Thus despite higher expression of FA oxidation genes, it is possible that FA oxidation might be depressed *in-vivo* leading to steatosis.

Some studies have shown CLA-induced down-regulation of genes related to mitochondrial β-oxidation (*CPT1*), and ω oxidation (*cyt P450* and *FMO3*) (Rasooly et al., 2007) .The expression of *CPT1*, *ACOX1,* and *FMO3* was decreased without any changes in hepatic lipogenic genes of lactating mice fed *trans*-10, *cis*-12 CLA (Kadegowda, A.K.G., Erdman, R.A., and Loor, J.J. Unpublished results). The variable responses among different studies can be attributed to the level and type of fat used in the experimental diet along with the physiological conditions of animal used in the experiment.

Effect of CLA on hepatic fatty acid composition.

Trans-10, *cis*-12 CLA-induced hepatic steatosis is characterized by changes in hepatic FA composition (Figure 2.3) (Belury and KempaSteczko, 1997; Sebedio et al., 2001; Chardigny et al., 2003; Kelley et al., 2004; Kelley et al., 2006; Kadegowda et al., 2010; Martins et al., 2011) similar to those induced during non-alcoholic fatty liver disease (NAFLD) (Puri et al., 2007). The hepatic FA composition in steatotic liver determines the extent of susceptibility of liver injury (Wang et al., 2006). The steatotic liver FA profile is characterized by substantial reductions in long chain polyunsaturated FA (LC-PUFA) concentrations; specifically that of arachidonic acid (C20:4n-6) (Araya et al., 2004). While linoleic (18:2n-6) and α-linolenic (18:3n-3) are unaltered, the concentrations of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) are decreased (Araya et al., 2004). The desaturation and elongation of linoleic and α-linolenic by desaturases (Δ^5-desaturase, Δ^6-desaturase) and elongases (ELOVL-2, ELOVL-3) are involved in synthesis of LC-PUFA (Eder et al., 2002). *Trans*-10, *cis*-12 CLA inhibits both Δ^5- and Δ^6-desaturase in HepG2 cells (Eder et al., 2002). A recent tracer study with [U-^{13}C] linoleic acid showed significant reduction in *n-6* PUFA synthesis by inhibition of elongation and desaturation in the liver homogenates of neonatal pigs (Lin et al., 2011). A decrease in arachidonic acid synthesis would alter eicososonoid metabolism and potentially reduce the synthesis of prostaglandin E2 (PGE_2) (Sugano et al., 1998), which is known to have protective effects on the liver (Lukivskaya et al., 2001).

Typical NAFLD is also characterized by increased *n-6:n-3* LC-PUFA ratio which favors lipid synthesis over lipid oxidation and secretion, leading to hepatic lipid

accumulation (El-Badry et al., 2007). *Trans*-10, *cis*-12 CLA reduces the *n-3* PUFA in liver (Kelley et al., 2006; Kelley et al., 2009) in addition to arachidonic acid. The *n-3* PUFA downregulate *SREBP-1c* and up-regulate *PPAR-α,* which regulates lipid oxidation *(CPT1, ACOX1)* and secretion *(ApoB100)*. A decrease in hepatic *n-3* PUFA would not only reduce lipid oxidation but increase lipogenesis, leading to hepatic steatosis (El-Badry et al., 2007). Although the *trans*-10, *cis*-12 CLA-induced responses in FA oxidation are variable in mice, consistently increased lipogenesis (Table 2.2) suggests a potential role for *n-3* PUFA. On the contrary, CLA feeding increased n-3 PUFA content and decreased n-6 PUFA in the rat (Li and Watkins, 1998; Eder et al., 2005) which could explain the differences in CLA effects between the two species. Although the exact mechanism of CLA action has not been elucidated, it was suggested that the metabolites of CLA, conjugated dienes (CD)18:3, CD20:3, and CD20:4 could compete with other PUFA at the level of formation and metabolism in liver and affect LC-PUFA synthesis (Banni et al., 2004).

CLA and SCD in hepatic lipid metabolism

In the adipose, there are some similarities between the effects of *trans*-10, *cis*-12 CLA and inhibition of *SCD1*. For example., reduced adiposity is observed with both dietary *trans*-10, *cis*-12 CLA and *SCD1* inhibition and one could speculate that the effects of *trans*-10, *cis*-12 CLA are mediated through *SCD1* as *trans*-10, *cis*-12 CLA decreases *SCD1* in adipose (Brown and McIntosh, 2003). However, a study with *SCD1*$^{-/-}$ mice showed that the anti-obesity effects of *trans*-10, *cis*-12 CLA were independent of *SCD1* gene expression and enzyme activity (Kang et al., 2004).

Unlike adipose, the effects of *trans*-10, *cis*-12 CLA are varied in liver (Table 2.2). While *trans*-10, *cis*-12 CLA decreased hepatic *SCD* activity *in-vitro* (Park et al., 2000), *in-vivo* studies report increased hepatic *SCD1* gene expression (Rasooly et al., 2007; Guillen et al., 2009). In contrast to *trans*-10, *cis*-12 CLA effects in mice, *SCD1*$^{-/-}$ mice showed increased insulin sensitivity, reduced hepatic lipogenic genes, up-regulated lipid oxidizing genes, increased hepatic saturated FA and unchanged hepatic *n-3* and *n-6* PUFA (Ntambi et al., 2002). *SCD1*$^{-/-}$ mice fed *trans*-10, *cis*-12 CLA showed reduced hepatic accumulation compared to wild-type mice (Kang et al., 2004) confirming that reduced *SCD1* expression decreases hepatic lipid accumulation (MacDonald et al., 2008). Liver specific *SCD1* knock-out decreased expression of *SREBP1* and *ChREBP* and their target genes thereby reducing hepatic lipogenesis (Miyazaki et al., 2007). In contrast, short-term inhibition of tissue specific hepatic *SCD* increased hepatic TG content and enhanced insulin signaling (Gutierrez-Juarez et al., 2006) but the long-term inhibition decreased hepatic steatosis (Jiang et al., 2005). The differences in responses observed in liver-specific knock-out versus complete *SCD* knock-out mice suggest that hepatic lipid metabolism is being affected by lipid metabolism in non-hepatic tissues (Flowers and Ntambi, 2008).

As *trans*-10, *cis*-12 CLA effects in mice are mostly associated with insulin resistance, increased hepatic *SCD1* expression is probably due to increased *SREBP-1c* expression. Hepatic steatosis due to *trans*-10, *cis*-12 CLA is also seen in the absence of insulin and is associated with reduced expression of *SCD1* and other lipogenic genes (Jourdan et al., 2009). These results indicate that the disturbances in hepatic lipid

metabolism caused by dietary *trans*-10, *cis*-12 CLA are mediated by multiple mechanisms (House et al., 2005) rather than through changes in *SCD1* alone.

Effect of CLA on mammary lipid metabolism

Among all the CLA isomers, the role of *trans*-10, *cis*-12 CLA has been extensively studied in relation to its effects on mammary lipid metabolism. *Trans*-10, *cis*-12 CLA has been clearly established as a potent inhibitor of milk fat synthesis (Baumgard et al., 2000). Abomasal infusion of 10 g/d of *trans*-10, *cis*-12 CLA reduced milk fat yield by 43% while no effects were observed on other milk components (Kadegowda et al., 2008). Similar findings were also observed with rumen-protected *trans*-10, *cis*-12 CLA supplemented over a period of 20 weeks (Perfield et al., 2002; Bernal-Santos et al., 2003).

The *trans*-10, *cis*-12 CLA is incorporated into milk fat and the transfer efficiency remains constant around ~22% across CLA dose ranges irrespective of a concomitant reduction of milk fat yield (de Veth et al., 2004). As a result of consistent uptake, the relationship between the dose of *trans*-10, *cis*-12 CLA and depression in milk fat yield is curvilinear (Bauman and Griinari, 2003). De veth et al.(2004) summarized data across experiments and demonstrated that a dose of *trans*-10, *cis*-12 CLA dose of 6 g/d produces a maximal response in milk fat reduction beyond which there is little decrease in milk fat synthesis (de Veth et al., 2004). The other major CLA isomer, *cis*-9, *trans*-11 CLA, has no effect on milk fat synthesis in lactating cows (Baumgard et al., 2000).

Mechanism

The effects of *trans*-10, *cis*-12 CLA-induced MFD alter the pattern of milk FA composition, providing the insight about the mechanism for the decrease in milk fat yield and composition (Bauman and Griinari, 2003). The reduction in milk fat yield involves significant reduction in FA of all chain lengths (Chouinard et al., 1999; Baumgard et al., 2002) however, the decrease in the yield of SMCFA are greater compared to LCFA (Chouinard et al., 1999; Bauman and Griinari, 2003) and the effects are pronounced at larger doses of *trans*-10, *cis*-12 CLA. Similar responses were observed in lactating mice although mouse milk has a lower proportion of *denovo* synthesized FA (Bauman et al., 2011). Thus *trans*-10, *cis*-12 CLA-induced alterations in milk FA composition and significant reductions in milk fat yield suggest that the mechanism may involve either reduced milk fat secretion and/or synthesis. Previous studies using pure CLA isomers indicate that milk fat synthesis is significantly reduced by *trans*-10, *cis*-12 CLA (Bauman et al., 2011). The infusion of *trans*-10, *cis*-12 CLA was accompanied by dramatic reductions of mRNA abundance of enzymes involved in *denovo* FA synthesis *(ACC, FASN)*, mammary uptake and intracellular transport of FA (*LPL, FABP)*, desaturation *(SCD)* and triglyceride synthesis (*GPAT, AGPAT)*(Piperova et al., 2000; Peterson et al., 2003). Lactation response to *trans*-10, *cis*-12 CLA is conserved across species including lactating rodents (Kadegowda et al., 2010). Similar findings were also observed *invitro* in bovine mammary cell line where mRNA abundance for *ACC, FASN, SCD* and *FABP* were reduced after 48 hours of incubation with *trans*-10, *cis*-12 CLA (Kadegowda et al., 2009, Peterson et al., 2004).

The consistent and coordinated suppression of mammary lipogenesis in both dairy cows and lactating rodents suggests the involvement of a central regulator of lipid synthesis. *SREBP1c* is the master regulator of mammary lipogenesis and mRNA abundance of its active nuclear fragment was decreased in response to *trans*-10, *cis*-12 CLA in dairy cows (Harvatine and Bauman, 2006; Gervais et al., 2009), mice (Kadegowda et al., 2010) and bovine mammary epithelial cells (Peterson et al., 2004). *Trans*-10, *cis*-12 CLA can downregulate the nuclear abundance of *SREBP-1* either by inhibiting proteolytic activation processing of *SREBP*-1 protein or inhibition of the *SREBP-1* gene transcription (Bernard et al., 2008). The addition of *trans*-10, *cis*-12 CLA in bovine mammary epithelial cells had no effect on *SREBP*-1 mRNA. However, the abundance of the active nuclear fragment was reduced, suggesting reduced proteolytic activation of *SREBP-1* precursor protein (Peterson et al., 2004). Similar findings were observed *invivo* (Loor et al., 2005). However, mammary expression of *SREBP-1* was decreased both *invivo* (Harvatine and Bauman, 2006; Kadegowda et al., 2010) and *invitro* (Kadegowda et al., 2009). The extent of *SREBP1* suppression is similar to the magnitude to the depression in milk fat yield during *trans*-10, *cis*-12 CLA-induced MFD (Gervais et al., 2009; Bauman et al., 2011). However the responses observed with *SREBP1* regulatory proteins like *INSIG1* are not consistent (Harvatine and Bauman, 2006; Kadegowda et al., 2010).

Dosage effect

The *trans*-10, *cis*-12 CLA dose used in rodent studies ranged between 0.3-1 percent to obtain maximum MFD, compared to 0.05% in dairy cows (Bauman et al., 2011). Larger doses of *trans*-10, *cis*-12 CLA in dairy cows have been shown to cause

generalized reduction in all milk component yield and dramatic increase in somatic cell count (Bell and Kennelly, 2003). Similar responses were observed in mice where larger doses (0.5%) negatively affected mammary development as it reduced ductal elongation and caused premature alveolar budding (Foote et al., 2010). These effects were associated with increased expression of inflammatory markers suggesting detrimental effects with excessive doses of *trans*-10, *cis*-12 CLA (Foote et al., 2010).

Table 2.1. Studies showing trans-10, cis-12 CLA-induced changes in body, adipose and liver weights and liver lipid concentration (Number of observations (mean percent change)

Species	Change	Body weight	Adipose tissue	Liver weight	Liver lipids
Mice[2]	Increase	-	-	24(92)	19(515)
	Decrease	21(31) [2]	29(666)	-	-
	No change	16	-	2	2
Rats[3]	Increase	-	-	-	1(25)
	Decrease	-	1(23)	-	4(19)
	No change	11	3	8	4
Hamsters[4]	Increase	-	-	8(20)	-
	Decrease	2(14)	11(20)	-	3(37)
	No change	11	2	2	5
Humans[5]	Increase	-	-	-	-
	Decrease	2	6	-	-
	No change	11	13	-	-

[2]Studies used:(Andreoli et al., 2009, Belury and KempaSteczko, 1997, Clement et al., 2002, Degrace et al., 2004, Degrace et al., 2003, DeLany and West, 2000, Foote et al., 2010, Halade et al., 2009, 2010, Ide, 2005, Jourdan et al., 2009, Kadegowda et al., 2010, Kelley et al., 2009, Liu et al., 2007, Nakanishi et al., 2004, Park et al., 1997, Park et al., 1999a, Park et al., 1999b, Poirier et al., 2005, Poirier et al., 2006, Rasooly et al., 2007, Takahashi et al., 2003, Tsuboyama-Kasaoka et al., 2000, Yanagita et al., 2005)
[3]Studies used:(Andreoli et al., 2007, Choi et al., 2004, Moya-Camarena et al., 1999, Purushotham et al., 2007, Tsuzuki et al., 2004)
[4]Studies used: (Bissonauth et al., 2006, de Deckere et al., 1999, Lasa et al., 2011, Macarulla et al., 2005, Miranda et al., 2009, Navarro et al., 2009, Simon et al., 2006, Tarling et al., 2009, Zabala et al., 2006)
[5]Studies used :(Basu et al., 2000, Benito et al., 2001, Berven et al., 2000, Gaullier et al., 2005a, Gaullier et al., 2005b, Kamphuis et al., 2003, Kreider et al., 2002, Malpuech-Brugere et al., 2004, Moloney et al., 2004, Mougios et al., 2001, Petridou et al., 2003, Racine et al., 2010, Riserus et al., 2002, Riserus et al., 2004, Taylor et al., 2006, Thom et al., 2001, Thrush et al., 2007, Whigham et al., 2004, Zambell et al., 2000)

Table 2.2. Studies showing *trans*-10, *cis*-12 CLA-induced changes in hepatic gene expression and circulating levels of insulin, adipokines and TNF-α. Genes are classified based on their ascribed function (↑, ↓, ↔; increase, decrease or no changes respectively)

	Mice[1]			Rats[2]			Hamsters[3]		
	↑	↓	↔	↑	↓	↔	↑	↓	↔
Lipogenesis									
ACC	5(126)[4]	-	1	-	-	-	1(99)	-	1
FASN	7(243)	-	1	-	1(50)	2	-	-	2
SCD1	2(150)	-	3	-	1(80)	-	-	-	-
SREBP1	3(53)	-	2	-	1(40)	4	-	-	3
PPAR-γ	2(200)	-	-	-	-	2	-	-	-
ME	5(205)	-	-	-	-	-	-	-	-
FA uptake, secretion and oxidation									
CPT1	4(107)	1(59)	1	-	-	-	-	-	2
ACO	5(117)	-	1	2(130)	-	4	-	-	2
PPAR-α	-	1(53)	-	1(125)	-	-	-	-	3
FAT/CD36	3(533)	-	-	-	-	-	-	-	-
LPL	-	-	1	-	-	-	-	-	1
Insulin, adipokines, and TNFα									
Insulin	12(2492)	1(29)	3	-	-	3	-	-	1
Adiponectin	-	6(77)	5	-	-	-	-	-	-
Leptin	-	10(71)	-	-	-	1	-	-	-
TNF-α	-	4(32)	1		1(44)	2	-	-	-

[1] Studies used: (Clement et al., 2002, Degrace et al., 2003, Guillen et al., 2009, Ide, 2005, Jourdan et al., 2009, Kadegowda et al., 2010, Lin et al., 2004, Liu et al., 2007, Rasooly et al., 2007, Takahashi et al., 2003, Yanagita et al., 2005)
[2] Studies used:(Choi et al., 2004, Moya-Camarena et al., 1999, Purushotham et al., 2007, Stringer et al., 2010, Tsuzuki et al., 2004)
[3]Studies used :(Lasa et al., 2011, Macarulla et al., 2005, Miranda et al., 2009, Tarling et al., 2009)
[4]Number of observations (mean percent change)

Table 2.3. Summary of literature studies on amelioration of CLA-induced hepatic steatosis

			% Added Dietary CLA				
Reference	n	days	CLA Mix	*t10,c12 CLA*	Treatment	Treatment Dose, %[1]	Observations
Nagao et al., 2008	3 - 6	28	2.0	0.95	Leptin	5 µg/d	↓ Hepatic steatosis, ↑ Insulin sensitivity,
Tsuboyama-Kasaoka et al., 2000	5-14	30	1.0	0.72	Leptin	5 µg/d	↑ Insulin sensitivity, Ameliorated hepatic steatosis
Purushotham et al., 2007	5	28	1.5	0.60	Rosiglitazone	10 mg/kg BW	↑ Insulin sensitivity, Prevented depletion of epididymal adipose tissue
Liu et al., 2007	10	42	2.0	1.00	Rosiglitazone	10 mg/kg BW	↓ Hepatic TG content, ↓ Hepatic lipogenesis, ↑ Serum leptin and adiponectin, Prevents lipodystrophy
Oikawa et al., 2009	7	28	3.0	0.98	Arachidonic acid	1, 2	↓ Induction of hepatic steatosis, ↑ liver PGE2, ↑ Epididymal adipose
Nakanishi et al., 2004	7	28	-	1.20	γ –Linoleic acid	5	↓ Hepatic steatosis, ↑ PGE_2
Kelley et al., 2009	10	56	-	0.50	Flax seed oil (α-Linoleic acid)	0.39	↓ Steatosis, ↑n-3 and n-6 PUFA in liver
Ide, 2005	7 - 8	22	1.0	0.50	Fish Oil	1.5,3,6	↑ Leptin and Adiponectin, ↓ Insulin, ↓ TG in liver, ↑ Fat pad

Ferramosca et al., 2008	10	105	1.0	0.50	Pine oil	7.5	Serum insulin levels stabilized over 3 weeks
Tsuboyama-Kasaoka et al., 2003	5 - 6	100	1.0	0.35	34% Dietary fat		Normal plasma insulin levels, ↑ Liver weight
Yanagita et al., 2005	6	28	2.0	0.74	DHA	0.5	↓ Fatty liver, ↓ FA synthesis, Plasma leptin and Adiponectin unaffected
Vemuri et al., 2007	10	56	-	0.50	DHA, EPA	0.5, 0.5	Prevented hepatic steatosis, Partially restored plasma leptin, Only DHA restored plasma adiponectin

[1]Percentage in the diet except wherever noted.

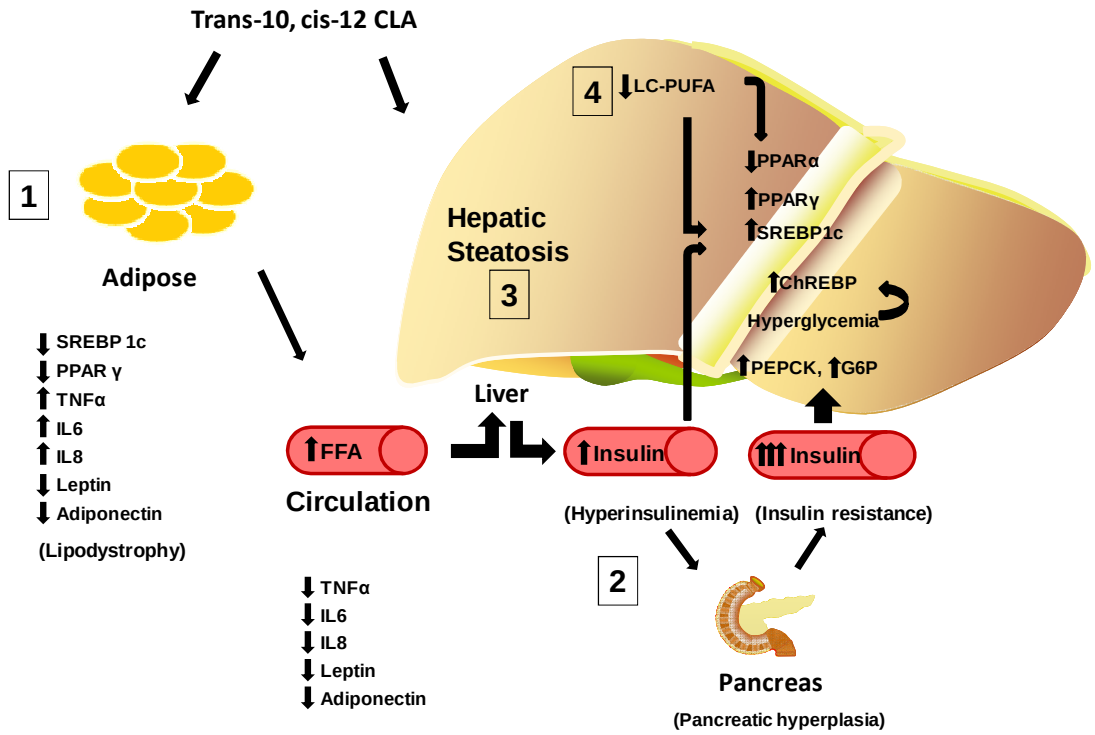

Figure 2.3. Current concepts in the pathways of trans-10, cis-12 CLA-induced hepatic steatosis

1. Adipose tissue lipodystrophy caused by increased proinflammatory cytokines and reduced adipokines leading to higher circulatory levels of free FA (FFA). **2.** Hyperinsulinemia induced by systemic insulin resistance. **3.** Alterations in hepatic lipid metabolism leading to hepatic steatosis. **4.** Alterations in hepatic FA composition. *SREBP1c, Sterol regulatory element binding protein; PPAR-γ, Peroxisome proliferator activated receptor- γ; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; IL-8, Interleukin-8; PEPCK, Phospho-enol pyruvate carboxykinase; G6P, Glucose 6-phosphatase; ChREBP, Carbohydrate response element binding protein; PPAR-α, Peroxisome proliferator activated receptor-α;* LC-PUFA, Long chain polyunsaturated FA.

Short-and medium-chain fatty acids (SMCFA)

While milk FA composition is different for every species, the pattern of FA composition must allow milk fat secretion at body temperature (Parodi, 1982; Dils, 1983). Ruminant milk is characterized by greater proportions of SMCFA (~50%) (Jensen, 2002) and may reflect the relative absence of PUFA. The physiological significance of SMCFA might be to maintain fluidity and to regulate the melting temperature of milk fat (Barbano and Sherbon, 1980). In addition, short-chain FA (SCFA) including acetate, propionate and butyrate are the major source of energy in ruminants, providing up to 80% of their maintenance energy requirements (Bergman, 1990). Propionate serves as a major precursor for glucose (Huntington et al., 1981), while acetate and butyrate are utilized for lipogenesis in adipose and mammary tissues. This contrasts with other species where glucose serves as the carbon source for SMCFA for *denovo* FA synthesis. In addition to energetic and nutritional contribution of SCFA, medium-chain FA (MCFA) including C12 and C14 have a strong potential in suppressing ruminal methanogenesis (Blaxter and Czerkaws, 1966).

Dietary SMCFA might play an important role during milk fat synthesis. As reviewed earlier, during MFD the proportions of SMCFA are reduced to a greater extent than LCFA, underscoring the importance of SMCFA in maintaining milk fat yield. Recently, abomasal infusion of butterfat, used as source for SMCFA, increased milk fat content and yield in lactating dairy cows even under normal conditions of milk fat synthesis (Kadegowda et al., 2008). To appreciate the role of SMCFA on milk fat synthesis, it is important to understand the absorption and metabolism of the respective FA.

Absorption and metabolism of SMCFA

Triglycerides containing SMCFA possess distinct physical and chemical properties characterized by low melting point, small molecular weight and water soluble properties which make their absorption and metabolism different from the TG containing LCFA (Dils, 1983; Marten et al., 2006).

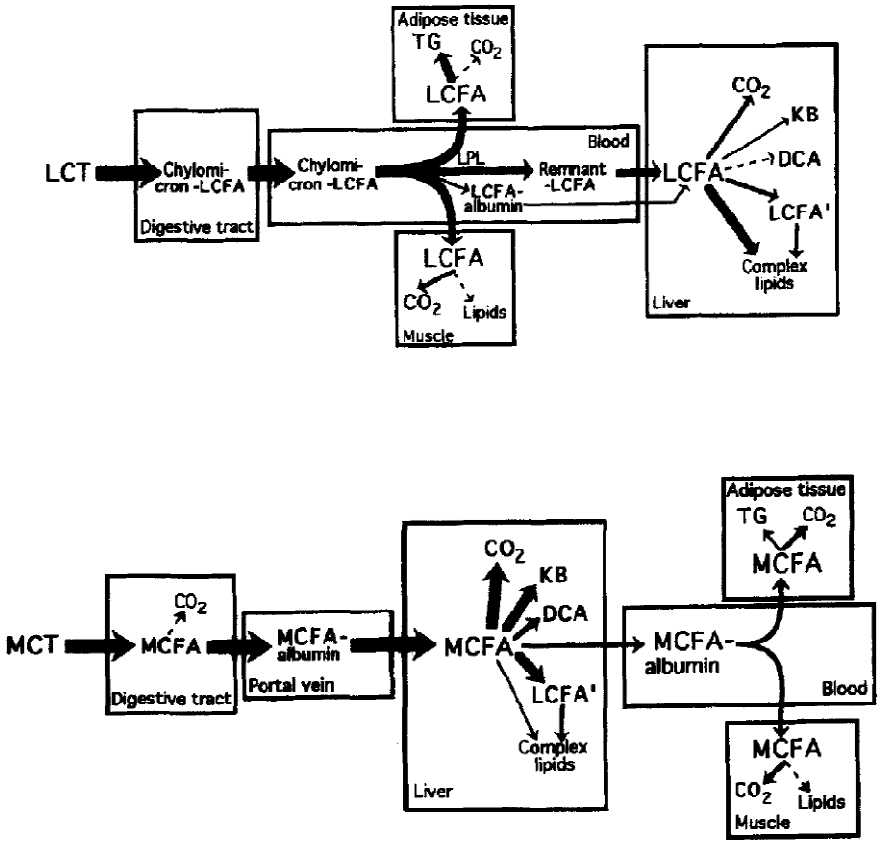

Figure 2.4. Transport, distribution and metabolic fate of exogenous FA according to their chain length (Bach et al.,1996)

In monogastric animals, pancreatic lipase completely hydrolyzes TG containing SMCFA, which further are transported directly to the liver via the portal vein as FA or monoglyceride (MG) and are preferentially metabolized as an energy source. SMCFA are

also more ketogenic than LCFA (Marten et al., 2006). The liver is able to produce ten times more CO_2 with C8 than with C16, which may exceed the capacity of liver Krebs cycle, directing acetyl-CoA towards ketone body formation (Bach and Babayan, 1982). On the contrary, pancreatic lipase incompletely hydrolyzes LCFA from TG resulting in the formation of LCFA, MG and DG. In addition, LCFA are absorbed by the intestinal mucosa, are incorporated into chylomicron TG, and reach the systemic circulation via the lymph system and preferentially get distributed to peripheral tissues before being utilized in liver (Bach and Babayan, 1982).

In ruminants, the short-chain VFA are produced by the anaerobic microbial fermentation of carbohydrates. The net absorption of VFA is dependent upon their concentration in rumen and amounts metabolized by the rumen wall. The rates of utilization by the rumen wall are butyrate > propionate > acetate (Stevens and Stettler, 1966; Kirat et al., 2006). Recently, the role of *monocarboxylate transporter-1 (MCT-1)* was demonstrated in the transepithelial transport and efflux of VFA across rumen epithelium towards the blood side (Kirat et al., 2006). Metabolic use of MCFA are not documented in dairy cows. However, greater intestinal digestibilities and reduced transfer efficiency of C12:0 and C14:0 in milk fat as compared to other LCFA (Dohme et al., 2004) suggest ruminal absorption and extensive hepatic utilization of the MCFA.

Milk fat responses to supplemental SMCFA

Milk fat responses to supplemental SMCFA are not well documented. Among earlier studies, Storry et al. (1969) observed milk fat responses to intravenous infusion of short-and medium-chain TG. Milk fat content and yield were significantly greater with C6, C12 and C14 TG, while no effects were observed for other short-chain TG. This was

probably due to extensive catabolism in extra mammary tissues. Further studies with individual MCFA (C12 and C14) showed inconsistent milk fat responses. Milk fat yield was significantly reduced with C14:0 (Steele and Moore, 1968; Odongo et al., 2007) while no effect was observed in others (Dohme et al., 2004; Hristov et al., 2011). Similarly, milk fat yield was reduced with C12:0 in some studies (Steele and Moore, 1968; Hristov et al., 2011,) while no effect was observed in others (Dohme et al., 2004). Coconut oil (CO), a rich source of C12 and C14 FA, was used at increasing levels in diet of lactating dairy cows but no effects were observed on milk fat content and yield (Storry et al., 1971). On the contrary, significant differences were observed in milk fat responses between unprotected and protected CO (Storry et al., 1974; Astrup et al., 1976). The protected form of CO not only improved rumen fermentation parameters but also increased milk fat content and yield as compared to unprotected CO (Storry et al., 1974; Astrup et al., 1976).

A recent *invitro* study in mammary epithelial cell lines showed that SMCFA (C4, C6 and C8) reduce *ACC* enzyme activity and increased the expression of *CD36* and *PPAR-γ* (Yonezawa et al., 2004). Octanoate (C8) stimulated the TG accumulation in a concentration-dependent manner and increased lipid droplet formation (Yonezawa et al., 2004). The inhibitory effects of C6 and C8 on *hexokinase* and *phosphofructokinase* and of C10 on *pyruvate dehydrogenase* enzyme further reflect the inhibitory effects of SCFA on milk fat synthesis (Heesom et al., 1992).

In lactating rats, MCT (medium-chain triglycerides, C8 and C10 reduced the rate of mammary lipogenesis by 82% (Agius and Williamson, 1980) and 57% (Souza and Williamson, 1993). Energy intake was also low due to rapid removal and hepatic

oxidation of MCFA, resulting in formation of ketone bodies, signaling a decrease in food intake.

The transfer efficiency of SMCFA into milk fat is very low as these FA are predominantly absorbed directly in the hepatic portal vein in contrast to LCFA which are mostly incorporated in to lipoprotein lipids and released in lymph (Grummer, 1991). Among SMCFA, the transfer efficiency of short-chain FA including C6 to C10 is minimal (Storry et al., 1969; Grummer and Socha, 1989) and dietary supplementation is not useful for increasing these FA in milk. However, C12 and C14 are transferred with relatively greater efficiency. The C12 represents the borderline in chain length where absorption shifts from rumen to intestine (Dohme et al., 2004). The transfer efficiency of C12 ranges between 18-26% (Rindsig and Schultz, 1974; Dohme et al., 2004; Hristov et al., 2009). The transfer efficiency of supplemented C14 is variable. While some have shown transfer efficiency in the range of 12-15% (Odongo et al., 2007; Hristov et al., 2009) others have shown greater transfer efficiency (39%) (Dohme et al., 2004). Similar findings were observed in rats where the transfer efficiency of C8 and C10 were less, as these FA were preferentially oxidized in liver and utilized for energy (Lavau and Hashim, 1978).

Hypothesis and study objectives

Based on the above literature the central hypothesis of the dissertation is "***the availability of denovo synthesized FA is the key limiting substrate for milk fat synthesis***". Following were the study objectives to test this hypothesis:

1. To determine the milk fat response to dietary supplementation of SMCFA in lactating dairy cows.
2. To determine the effects of increased availability of SMCFA during CLA-induced MFD on mammary lipid metabolism.
3. To study mammary and hepatic lipid metabolism in response to a *PPAR-* γ agonist during CLA-induced MFD in lactating mice.

The following figures summarize the milk fat synthesis process and the experimental approaches used to test the hypothesis.

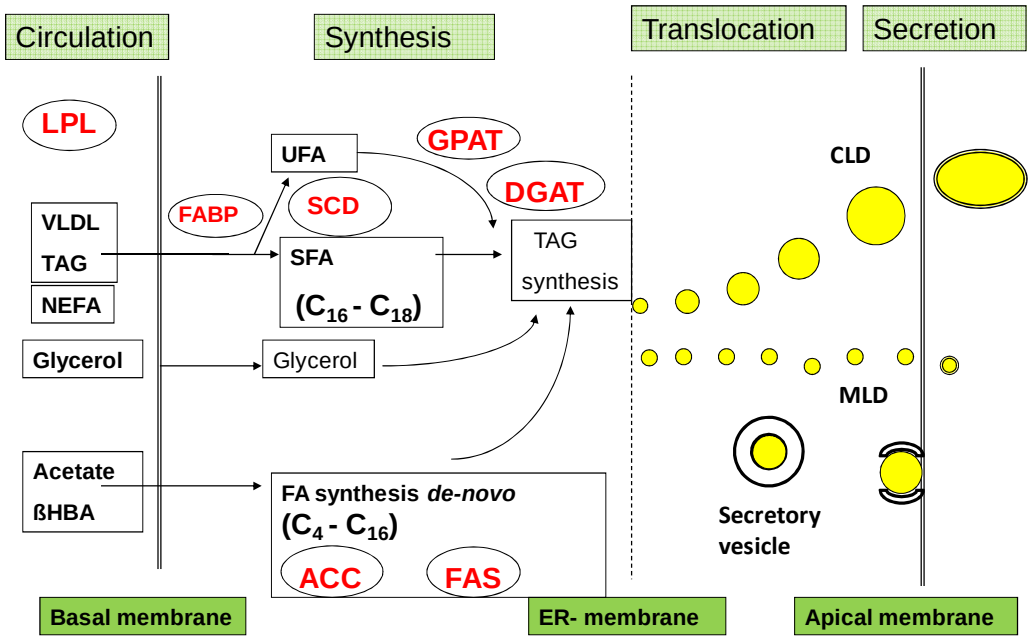

Figure 2.5. The basics of milk fat synthesis (Modified from Baumgard, L. H., 2002)

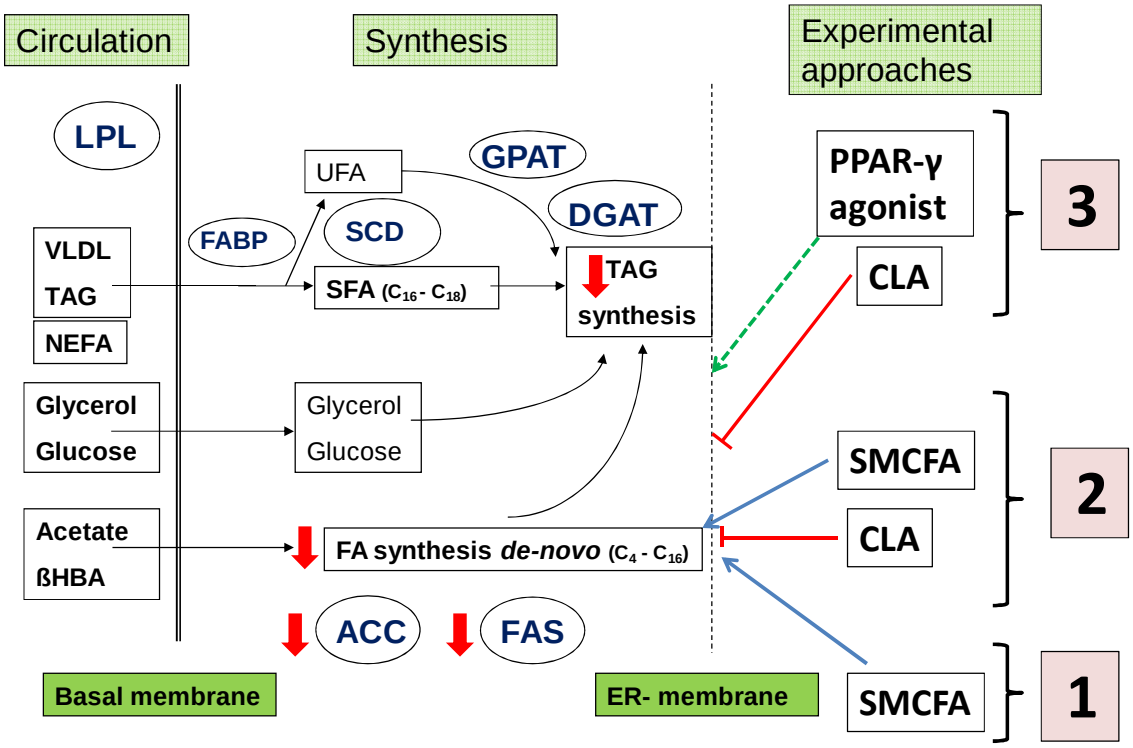

Figure 2.6. The experimental approaches used to test the potential limitation of *denovo* synthesized fatty acids for milk fat synthesis (Modified from Baumgard, L. H., 2002)

1. To determine the milk fat response to dietary supplementation of SMCFA in lactating dairy cows.
2. To determine the effects of increased availability of SMCFA during CLA-induced MFD on mammary lipid metabolism.
3. To study mammary and hepatic lipid metabolism in response to a *PPAR-* γ agonist during CLA-induced MFD in lactating mice.

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Chapter 3: EXPERIMENT 1

Milk fat responses to dietary supplementation of short-and medium-chain fatty acids in lactating dairy cows[1]

[1] D. Vyas, B. B. Teter, and R. A. Erdman. 2010. Milk fat responses to dietary short and medium chain fatty acids in lactating dairy cows. J. Dairy Sci. Vol. 93, E-Suppl. 1:444.

ABSTRACT

Short-and medium-chain fatty acids (SMCFA), which are synthesized *denovo* in the mammary gland, are reduced to a much greater extent than the long-chain fatty acids during diet-induced milk fat depression. Our hypothesis was that SMCFA are limiting for milk fat synthesis even under conditions when milk fat is not depressed. Our objective is to test the potential limitation of SMCFA on milk fat synthesis via dietary supplementation. Sixteen lactating Holstein cows (107 ± 18 DIM) were fed a corn silage-based total mixed ration. Cows were randomly assigned to groups of 4 per pen and supplemented with one of 4 dietary fat supplements (600g/d) supplied in a 4x4 Latin square design with 21-d experimental periods. Treatments consisted of fat supplements containing mixtures of calcium salts of long-chain fatty acids (Megalac®; M) and a SMCFA mixture (S) (C8- 3.3%, C10- 7.6%, C12- 9.85%, C14- 32.12% and C16- 47.11%) that contained 0, 200, 400, and 600 g/d S substituted for M (S0, S200, S400, and S600, respectively). No treatment effects were observed for dry matter intake, and fat-corrected milk. However, milk yield was linearly reduced with S600. Milk fat increased linearly by 0.17, 0.25, and 0.33 percentage units for the respective S treatments. However, fat yield peaked at S200 and milk protein concentration and yield was significantly reduced at the higher S levels due to a linear trend towards reduced milk yield in the S600 treatment. In conclusion, SMCFA supplementation linearly increased milk fat concentration but reduced milk production at the higher levels of supplementation. The dietary inclusion of SMCFA had no effects on milk fat yield.

INTRODUCTION

In 2000, the USDA Federal Milk Marketing System adopted a multiple component pricing (MCP) system that changed the milk payment system for dairy producers to one based primarily on the amounts of milk components produced rather than milk volume. Among the milk components, milk fat and milk protein are the most valuable as compared with other solids (lactose and minerals) (USDA-NASS, 2009). Milk fat responses to dietary manipulations are large as compared to other milk components (Sutton, 1989). This signifies the importance of understanding the metabolic regulation of milk fat synthesis. In turn, understanding regulation of milk fat synthesis would further help in developing practical guidelines for dairy farmers to better control the quality and quantity of milk fat produced in relation to market demands.

Milk fat consists primarily of triglycerides (TG**)** which include a glycerol backbone and three ester-linked FA. The basic mechanism of milk fat synthesis depends on two general sources of FA; i.e. *denovo* FA synthesis in the mammary gland, and transfer of preformed FA from TG in the blood. The SMCFA including FA from C4-C14 and half of C16 are synthesized *denovo* while LCFA either originate from diet or are mobilized from adipose TG as preformed FA (Moore and Steele, 1968). Dietary manipulations can reduce milk fat by 46% (Piperova et al., 2000). During diet-induced milk fat depression (MFD) the proportion of *denovo* synthesized FA are reduced to a much greater extent than LCFA (Banks et al., 1984; Loor and Herbein, 1998). The alterations in FA composition are attributed to reduced acetyl-CoA carboxylase (ACC), a rate limiting enzyme for *denovo* FA synthesis (Mellenberger et al., 1973), and fatty acid

synthase (FAS) enzyme activity (Piperova et al., 2000). Under these situations *denovo* FA synthesis might be limiting for milk fat synthesis and the provision of dietary SMCFA in the proportion of *denovo* synthesized FA might rescue MFD. However, abomasal infusion of butterfat, used as a source of SMCFA, increased milk fat yield even under normal conditions of milk fat synthesis (Kadegowda et al., 2008). This led to our hypothesis that SMCFA might be limiting even during normal conditions of milk fat synthesis. The objective of our experiment was to test the potential limitation of SMCFA by dietary supplementation of a FA mix containing SMCFA in incremental doses and observing their effects on milk fat responses in lactating dairy cows.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

All procedures for this experiment were conducted under a protocol approved by the University of Maryland Institutional Animal Care and Use Committee. Sixteen Holstein dairy cows in mid lactation (107 ± 18 DIM) were used in 4x4 Latin square design balanced for carry over effects. Treatments were as follows: 1) S0 - Fed basal diet with 600 g/d of Megalac (M); 2) S200 - Fed basal diet with 400 g/d M and 200 g/d SMCFA mix (S); 3) S400- Fed basal diet with 200 g/d M and 400 g/d S; and 4) S600- Fed basal diet with 600 g/d of S and no M.

The dietary S consisted of a mixture of C8, 3.3 %; C10, 7.6 %, C12, 9.9 %; C14, 32.1 % and C16, 47.1 % as free FA (Sigma-Aldrich, St. Louis, MO) corresponding to the proportions of *denovo* synthesized FA in milk fat. Short-chain FA including C4 and C6

could not be added in the FA mixture because of their volatile nature. The fat supplements were thoroughly mixed with concentrates before being incorporated into the basal diet to ensure homogenous mixing. The relative proportions of FA in S are shown in Figure 3.1.

Experimental Procedure

Experimental periods were 3 wk. Control diet was fed during the first week of each period to reduce carryover effects. This was followed by 2 wk of treatment diets. Four cows were randomly allotted to each pen which included access to sawdust bedded freestalls. All cows within each pen were group fed. The basal diet contained 56% forage and 44% concentrate (DM basis) to meet NRC (2001) nutrient specifications for a 600-kg cow producing 40 kg of milk containing 3.7% milk fat and 3.1% milk protein. Ingredient and chemical composition of the basal diet is given in Table 3.1. Forage and ingredient DM were measured weekly, and the TMR was adjusted accordingly to maintain a constant forage-to-concentrate ratio on a DM basis. Amounts of feed offered and refused were recorded once daily at 0800h.

Milk Sampling and Component Analysis

Cows were milked twice daily at 0700 and 1700 h, and milk production was recorded using automated milk recording system at each milking. Two sets of milk samples were collected from the last 6 consecutive milkings of wk 3 of each experimental period. One set was collected with preservative for milk fat, protein, and somatic cell count (SCC) analysis by infrared analysis (Foss Milk-O-Scan, Foss Food Technology Corp., Eden Prairie, MN) and milk urea nitrogen (MUN)

by direct chemical analysis (Bentley Chemspec, Bentley Instruments, Inc., Chaska, MN). A second set of milk samples were composited and frozen at -20°C for subsequent analysis of individual FA.

FA Composition

Milk fat was extracted using a modified Folch procedure (Christie, 1982). The FA methyl esters (FAME) were prepared by mild transesterification with 1.4 mol/L of H_2SO_4 in methanol (Christie, 1982). The FAME were analyzed using an Agilent 5890 GC (Agilent Technologies, Santa Clara, CA) equipped with a Supelco 2560 capillary column (30 m x 0.25 mm id, Supelco, Bellefonte, PA) and a flame ionization detector. The column was maintained at 150°C for 5 min followed by ramp of 1.6°C/min to 180°C, then at 1.4°C/min to 190°C and finally holding the temperature at 190°C for 10 min. Nitrogen was used as carrier gas with a linear velocity of 25 cm/s and split ratio of 1:100. The injection port and detector were maintained at 250°C. Detector airflow was 222 mL/min, and hydrogen flow was 36 mL/min. Helium make-up gas was used at 80 mL/min. Individual FA and 18:1 isomers were identified using GLC-463 standard mixture (Nu-Chek Prep Inc., Elysian, MN).

Short-and medium-chain FA were analyzed as butyl esters (FABE), which were mathematically converted to FAME and normalized to the FAME chromatogram (Gander et al., 1962). The original FABE procedure was modified as follows. Milk samples were heated in screw-capped test tubes at 80°C for 1 h in the presence of 1.4 N H_2SO_4 in butanol followed by extraction with hexane in the presence of saturated KCl and distilled water. Samples were then centrifuged at 500 x *g* for 5 min at room temperature. An

aliquot of the upper hexane layer was injected directly into a Hewlett-Packard 5880 gas chromatograph equipped with a split injector, a flame ionization detector, and a 25 m x 0.2 mm fused silica capillary column coated with HP1 (Hewlett Packard, Avondale, PA). Helium was used as the carrier gas at a flow rate of 2 mL/min with a split ratio of 45:1. Injector and detector temperatures were set at 250°C, while column temperature started at 130°C. Ramp was set at 6°C/min to 290°C, followed by 4°C/min to 260°C and finally holding at 260°C for 20 min. Standard mixtures, including GLC-60 (Nu-check Prep, Inc., Elysian, MN) were converted to FABE to aid in the identification and quantification of components.

Statistical Analysis

Dry matter intake, milk production, milk components, and milk FA composition data for cows within each pen were summarized by experimental week and the pen average per cow from wk 3 was used as the experimental unit in the statistical analyses (SAS, Version 9.2, SAS Institute, Cary, NC). Transfer efficiency of individual FA were calculated as FA output in milk divided by the FA intake. The statistical model included treatments as fixed effects, whereas pen and period were used as random effects. Treatment effects were tested using linear and quadratic orthogonal contrasts. Significance for all effects was declared at $P \leq 0.05$.

RESULTS

The nutrient composition of the TMR and FA profile of individual dietary ingredients are presented in Tables 3.1 and 3.2, respectively. The total amount of dietary fat supplemented with the basal diet was 509, 539, 569 and 599 g with S0, S200, S400,

and S600 treatments, respectively (Table 3.3). The differences reflect substitution of free FA for Ca salts of FA in Megalac®. The treatments were designed to provide increasing amounts of SMCFA including C8, C10, C12 and C14 while keeping C16 constant in all groups.

Production responses to fat supplement treatments are shown in Table 3.4. Treatments had no detectable effects on DMI, NE_L intake, and 3.5% FCM. However, there was a linear reduction ($P < 0.03$) in milk yield with increasing S. Milk production efficiency (3.5% FCM/DMI) was linearly increased with S treatments, reflecting a combination of the small but nonsignificant decreases in DMI, and increases in 3.5% FCM that were observed with S200 and S600 relative to S0.

Milk fat content increased linearly in a dose-responsive manner by 4, 7, and 9% with S200, S400, and S600, respectively ($P < 0.05$). Milk fat yield was numerically higher with S treatments and peaked at S200 (1,709 g/d). However, the differences were not significant ($P = 0.28$). Milk protein content and yield increased with S200 (3.12%, 1,373 g/d) but were linearly ($P < 0.01$) reduced at higher S levels (S400 and S600).

Concentrations (g/100 g of FAME) and yield (g/d) of individual FA in milk are shown in Tables 3.5 and 3.6, respectively. The content of individual short-chain FA (SCFA, C4-C12) were not altered. However the proportions of medium-chain FA (MCFA) were increased with S treatment. Myristic acid (C14) content was increased ($P < 0.001$) by 8, 19, and 26% and yield was increased by13, 24 and 32% with S200, S400 and S600 respectively. Myristoleic acid (C14:1) concentration also increased ($P < 0.01$) linearly with S treatment. Palmitic acid (C16) concentration was increased ($P < 0.01$) by

5 and 7% with S400 and S600, respectively. However the yields of C16 were not affected. Total C18 content was reduced ($P < 0.01$) by 13 and 24% at higher S levels (S400, S600). Further, total C20 FA content was reduced ($P < 0.01$) by 24 % with S600. Total saturated FA content was increased ($P < 0.001$) by 6% while monounsaturated FA (MUFA) were reduced by 10 and 12% ($P < 0.001$) with S400 and S600, respectively.

The transfer efficiencies for different SMCFA are shown in Figure 2.2. The transfer efficiencies were 15.2, 15.9, and 1.4 for C8; 19.3, 2.9, and -2.1 for C10; 28.7, 16.7, and 11.2 for C12:0; and 34.6, 31.9, and 28.4 for C14:0 for S200, S400, and S600, respectively. These values were calculated as the amount of FA excreted in the milk fat expressed as the percentage of the amount added to the TMR by subtracting the SMCFA treatment yields from the S0 treatment.

DISCUSSION

The purpose of this study was to evaluate the potential limitation of SMCFA on milk fat synthesis in lactating dairy cows. Treatments included incremental amounts of SMCFA substituted for M to keep diets isolipidic. However, the actual amounts of fat supplemented varied with each treatment due to differences in the FA content of individual supplements (85%, M; 98%, C8; 97%, C10; 98%, C14; 99%, C16). Never-the-less, the differences were not large enough to significantly change the interpretation of data. In addition, as desired the amount of SMCFA supplemented increased, whereas C16 intake was constant in all diets.

The DMI was similar for all treatments but was 1 kg/d less in the S600 treatment. While the effects of SCFA on feed intake are not well documented, MCFA can reduce

feed consumption when supplemented at high dietary concentrations. For instance DMI was significantly reduced with lauric acid supplemented at 4% of diet DM (Dohme et al., 2004). Increased feed refusals were also reported by feeding myristic acid at 5% of diet DM (Odongo et al., 2007) and with coconut oil (CO) containing higher concentrations of lauric and myristic acid (Storry et al., 1974). Medium chain FA was shown to disrupt rumen metabolism by reducing the number of protozoa, depressing fiber degradability, and subsequently depressing intake (Dohme et al., 2001). However, due to low dietary proportions of MCFA in the present study, significant interaction with the rumen microbes can likely be ruled out as the amount required to disrupt microbial population is thought to be 4% of DMI when MCFA are supplemented with a TMR (Hristov et al., 2009). The milk yield for high-producing dairy cows is limited by DMI (Allen, 2000). Therefore, the numerical reduction in milk yield and 3.5% FCM at higher S levels might reflect the nonsignificant decrease in DMI. A similar response was observed in dairy cows with intraruminal infusion of lauric acid and CO (Hristov et al., 2009).

Milk Fat and FA Composition

Previous studies have reported variable milk fat responses to individual SMCFA supplemented either as TG or free FA. Intravenous infusion of TG containing SCFA (C3, C4, C6, C8, C9, and C10 showed no changes whereas infusion of MCT increased milk fat yield (Storry et al., 1969). Similarly MCFA either reduced (Hristov et al., 2011) or showed no effect (Hristov et al., 2009) on milk fat yield. Similarly CO either improved (Storry et al., 1971) or had no effect (Hristov et al., 2009) on milk fat yield. Astrup et al., (1978) found that rumen-protected coconut oil increased milk fat percent, however, unprotected CO decreased fat percent. Milk fat output was improved in the present study

although the effects were not significant. This effect is probably not related to energy consumption as energy intake was similar among all the treatments. Effects of SMCFA are more apparent when the yields of individual FA are considered. The yield and content of SCFA were maintained and MCFA were increased with S treatments. The availability of palmitic acid was similar for all treatments, however, its secretion numerically improved with S treatments. This effect could be attributed to chain elongation of dietary SMCFA as similar response was observed with intravenous infusion of TG containing SCFA (Storry et al., 1969). The availability of SMCFA along with palmitic acid can affect milk TG synthesis (Hansen and Knudsen, 1987).

Milk fat is comprised of 95-98% TG (Jensen, 2002) and mammary TG synthesis involves FA esterification on 3 carbons of the glycerol backbone. Palmitic acid is an important precursor for initiating acylation at the sn-1 position, forming sn-1 lysophosphatidic acid (Hansen and Knudsen, 1987), which subsequently can be used as a substrate for myristyl-, oleoyl-, and stearoyl-CoA acylation (Kinsella and Gross, 1973). Short-chain FA are required for the rate-limiting step of esterification at the sn-3 position which is catalyzed by diacylglycerol acyl transferase (DGAT) (Parodi, 1979) The increased availability of palmitic acid might have increased incorporation of SMCFA during TG synthesis as reflected in milk fat composition with higher yield of *denovo* synthesized FA (DNFA). However, the responses were not significant due to reduced milk yield at higher levels of SMFCA supplementation. The yield of other LCFA including C18 and C20 may have been reduced due to dietary supply as a result of substitution of S for Ca salts of FA or substrate competition with SMCFA during TG

synthesis (Storry et al., 1969). The increased myritstoleic acid yield suggests increased mammary stearoyl-CoA desaturase activity with S treatments (Hristov et al., 2011).

Transfer Efficiency

Calculated apparent transfer efficiencies of individual FA are affected by their absorption and metabolism. The lower transfer efficiency of SCFA observed at higher levels of supplementation might be due to its preferential utilization by extra-mammary tissues. Short-chain FA can be absorbed directly from the digestive tract into the portal vein and can be preferentially oxidized in the liver (Souza and Williamson, 1993). Previous reports in mice and dairy cows have shown extensive hepatic oxidation and reduced incorporation of C8 and C10 in extra hepatic tissues (Lavau and Hashim, 1978; Storry et al., 1969; Souza and Williamson, 1993). Among the MCFA, 11-28% of dietary lauric acid was transferred in milk fat. This transfer efficiency is comparable to previous reports showing either 18% (Hristov et al., 2009) or 24-26% (Rindsig and Schultz, 1974; Dohme et al., 2004) of dietary lauric acid secreted in milk. Dietary myristic acid was transferred to milk at a rate between 28 and 35%, which is consistent with 39% reported previously (Dohme et al., 2004). However, these efficiencies are much greater than those reported by Hristov et al. (2009) and Odongo et al. (2007) of 15% and 12%, respectively. The reasons for such discrepancies are not clear. However, the amounts of C14 supplemented were much higher in previous studies (Odongo et al., 2007; Hristov et al., 2009), which might have reduced transfer efficiency due to extramammary utilization of the supplemented FA.

CONCLUSION

This is the first animal trial studying the effects of increasing amounts of dietary SMCFA on milk fat synthesis. Results indicate that increasing SMCFA linearly increased milk fat percentage. However, no effect was observed for milk fat yield due to depressed DMI and reduced milk yield. Further research is required to determine the mechanism by which these FA affect milk fat synthesis under conditions where DMI is maintained.

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Table 3.1. Ingredient and chemical composition of the basal diet fed to lactating cows

Item	DM %
Ingredient	
Corn silage	36.71
Alfalfa Hay	19.59
Corn grain, Ground	19.21
Citrus pulp	4.66
Soybean meal	16.22
Corn gluten meal (60%)	0.35
Limestone	0.73
Calcium phosphate	0.42
Magnesium oxide	0.17
Sodium bicarbonate	0.59
Potassium bicarbonate	0.11
Dynamate	0.11
Salt	0.39
Trace minerals and Vitamins[1]	0.18
Megalac	0.57
Chemical composition	
DM, %	59.64
CP, %	15.71
RUP, %	36.62[2]
ADF, %	21.51
NDF, %	35.52
NE_L, Mcal/kg	1.48[2]
Ca	0.84
P	0.48
Mg	0.29
K	1.85
Na	0.36
FA composition, g/100 g of FA	
14:0	0.59
16:0	21.86
16:1	1.60
18:0	3.65
18:1	19.52
18:2	33.27
18:3	11.90
Other	7.61

[1]Trace mineral and vitamin mix combined which provided an additional 0.76 mg/kg Co, 10 mg/kg Cu, 5.5 mg/kg Fe, 0.64mg/kg I, 37 mg/kg Zn, 0.33 mg/kg Se, 3,526 IU/kg vitamin A, 1,175 IU/kg vitamin D, and 22 IU/kg vitamin E to the diet DM. [2]Calculated value based the estimated TDN from feed analysis at 3X maintenance intake using NRC (2001) prediction equations.

Table 3.2. Fatty acid composition of different feed ingredients

Fatty acid	Alfalfa Hay	Corn Silage	Corn	Soybean meal	Citrus Pulp
	g/100 g of FAME[1]				
12:0	2.19	0.57		0.06	0.43
12:1	0.95	0.51			
13:0	0.39	0.33			
13:1	0.34	0.32			
14:0	0.71	0.83	0.07	0.17	0.51
14:1	0.81	0.61			
15:0	0.46	0.26		0.08	0.13
15:1	0.28	0.26			0.17
16:0	19.71	22.43	13.62	18.15	26.01
16:1	1.79	3.03	0.14	0.11	0.82
17:0	0.31	0.47	0.08	0.17	0.32
17:1	0.52	0.69			0.18
18:0	3.43	3.79	1.98	4.57	4.39
18:1 *(c9)*	3.89	21.64	26.63	10.58	20.39
18:1 *(c11)*	0.32	0.84	0.62	1.37	2.66
18:2	18.03	24.31	53.13	54.24	33.82
19:0	0.16				
20:0	1.20	1.44	0.45	0.20	0.49
18:3	34.19	8.0	1.61	9.05	7.22
20:1	0.12		0.39		
20:2					
20:3		0.30			
22:0	2.10	1.92	0.19	0.44	0.45
22:1	0.50	0.57		0.15	
24:0	1.24	2.26	0.24	0.29	0.69
24:1	2.17	0.36			
Ether extract, % of DM	3.64	2.56	4.58	3.85	5.80

[1]Fatty acid methyl ester

Table 3.3. Amount of fatty acid supplemented, g/d

Fatty acid	Treatments			
	S0	**S200**	**S400**	**S600**
8:0	0.5	7.5	14.4	21.3
10:0	0.2	15.0	29.9	44.7
12:0	2.9	21.5	40.1	58.8
12:1		0.2	0.5	0.7
14:0	6.6	68.5	130.3	192.2
16:0	273.6	276.0	278.4	280.8
16:1	0.7	0.4	0.2	
17:0	0.7	0.5	0.2	
18:0	23.4	15.8	8.2	0.5
18:1 (c-9)	166.3	110.9	55.4	
18:2	30.8	20.5	10.3	
20:0	1.7	1.2	0.6	
18:3	0.6	0.4	0.2	
20:1	0.6	0.4	0.2	
Total (g)	508.5	538.7	568.8	599.0

Table 3.4. Least squares means for production responses to increasing short-and medium-chain fatty acids

	Treatments					*P*-value[1]		
Item	S0	S200	S400	S600	SEM	SMCFA	Linear	Quadratic
DMI, kg/d	26.6	26.4	26.5	25.5	0.53	0.24	0.12	0.26
NE_L, Mcal/d	39.3	39.3	39.3	37.8	0.75	0.20	0.10	0.20
Milk, kg/d	43.6	43.9	42.7	41.1	1.14	0.08	0.03	0.18
3.5% FCM, kg/d	45.0	46.7	46.0	44.8	1.83	0.36	0.67	0.12
3.5% FCM/DMI	1.70[b]	1.76[a]	1.73[ab]	1.75[a]	0.04	<0.01	0.03	0.16
Milk fat, %	3.76 [b]	3.92 [ab]	4.01 [a]	4.10 [a]	0.16	<0.01	<0.01	0.34
Milk fat, g/d	1614	1709	1694	1663	89.9	0.28	0.40	0.11
Milk protein, %	3.10 [ab]	3.12 [a]	3.09 [ab]	3.06 [b]	0.05	0.03	0.01	0.07
Milk protein, g/d	1351 [ab]	1373 [a]	1318 [ab]	1257 [b]	41.5	0.05	0.02	0.13
MUN[2], mg/dL	10.54[a]	10.16[b]	10.49[a]	10.02[b]	0.44	<0.01	<0.01	0.29
SCC[3]. 1000's/mL	115[b]	118[b]	148[a]	129[ab]	2.3	<0.01	0.01	0.1

[a–d] Means within a row with different superscripts differ ($P < 0.05$).
[1] Probability of a significant effect of SMCFA or of a linear or quadratic effect of increasing amounts of dietary SMCFA
[2] Milk urea nitrogen
[3]Somatic cell counts

Table 3.5. Least squares means for fatty acid composition (%) of milk from cows in response to S0, S200, S400, and S600 treatments

	Treatments					*P*-value		
Fatty acid	S0	S200	S400	S600	SEM	Treatment	Linear	Quadratic
	g/100g FAME							
4:0	3.22	3.24	3.39	3.20	0.19	0.66	0.26	0.59
6:0	2.07	2.07	2.15	2.06	0.12	0.74	0.30	0.84
8:0	1.31	1.31	1.33	1.26	0.06	0.69	0.29	0.64
10:0	3.20	3.21	3.12	2.98	0.14	0.42	0.38	0.16
12:0	3.87	4.02	4.11	4.10	0.14	0.50	0.72	0.21
14:0	11.10[a]	11.99[b]	13.22[c]	14.04[c]	0.27	<0.01	0.18	<0.01
14:1	1.65[a]	1.74[ab]	1.94[b]	2.17[c]	0.07	<0.01	0.01	<0.01
15:0	1.16	1.11	1.19	1.19	0.08	0.61	0.82	0.24
15:1	0.25	0.25	0.23	0.25	0.03	0.81	0.56	0.43
16:0	34.80[b]	35.80[ab]	36.68[a]	37.33[a]	0.65	<0.01	0.66	<0.01
16:1 (*n9*)	0.52	0.50	0.51	0.52	0.02	0.74	0.43	0.87
16:1 (*n7*)	2.50	2.52	2.65	2.95	0.20	0.07	0.14	0.03
17:0	0.45	0.43	0.43	0.42	0.01	0.17	0.84	0.08
18:0	6.72[a]	6.76[ab]	6.19[ab]	5.86[b]	0.28	0.05	0.38	<0.01
18:1 (*c9*)	19.16[a]	18.14[a]	16.37[b]	15.18[b]	0.48	<0.01	0.17	<0.01
18:1 (*t9*)	1.40	1.22	1.67	1.38	0.18	0.71	0.29	0.81
18:1 (*c11*)	0.32[a]	0.30[ab]	0.28[ab]	0.26[b]	0.01	0.05	0.72	0.01
18:1 (*t11*)	0.68	0.63	0.65	0.64	0.05	0.65	0.99	0.71
18:2 *(t,t)*	0.34[a]	0.34[a]	0.28[b]	0.25[b]	0.02	<0.01	0.28	<0.01
18:2 (*n6*)	1.98	2.17	1.81	1.78	0.21	0.08	0.60	0.02
18:3 (*n3*)	0.23[a]	0.21[a]	0.19[ab]	0.18[b]	0.01	<0.01	0.42	<0.01

Total 18	30.83 [a]	29.78 [a]	26.94 [b]	25.51 [b]	0.86	<0.01	0.24	<0.01
19:0	0.12	0.14	0.12	0.13	0.02	0.85	0.93	0.67
20:0	0.13 [a]	0.14 [a]	0.12 [ab]	0.09 [b]	0.01	0.02	0.04	0.01
20:1(*c8*)	0.13	0.10	0.11	0.09	0.03	0.65	0.80	0.43
20:1 (*c11*)	0.17	0.16	0.14	0.15	0.02	0.53	0.56	0.18
21:0	0.15 [a]	0.13 [ab]	0.13 [ab]	0.11 [b]	0.01	0.01	0.38	0.02
20:3 (*n3*)	0.14	0.14	0.13	0.11	0.01	0.40	0.49	0.13
20:4 (*n6*)	0.19 [a]	0.17 [ab]	0.16 [ab]	0.13 [b]	0.02	0.02	0.32	0.01
Total 20	0.76 [a]	0.71 [ab]	0.66 [ab]	0.58 [b]	0.05	<0.01	0.26	<0.01
DNFA[2]	42.17[c]	43.75[bc]	45.65[ab]	46.26[a]	0.77	<0.01	0.92	<0.01
MUFA[3]	26.78[a]	25.57[ab]	24.03[b]	23.57[c]	0.69	<0.01	0.94	<0.01
PUFA[4]	2.74[ab]	2.88[a]	2.44[ab]	2.34[b]	0.23	0.01	0.44	<0.01
SFA[5]	68.30[b]	70.37[ab]	72.17[a]	72.73[a]	0.95	<0.01	0.89	<0.01

[a–c] Least squares means within a row with different superscripts differ ($P < 0.05$).
[1] Fatty acid methyl ester
[2] *Denovo* FA (DNFA) includes C4:0, C6:0, C8:0, C10:0, C12:0, C14:0 and half of C16 yield.
[3] Mono-unsaturated FA
[4] Poly-unsaturated FA
[5] Saturated FA

Table 3.6. Least squares means for yield (g/d) of fatty acids in milk from cows in response to S0, S200, S400, and S600 treatments

	Treatments					*P*-value		
Fatty acid	S0	S200	S400	S600	SEM	Treatment	Linear	Quadratic
	g of FAME[1] / day							
4:0	48.68	51.38	53.36	50.92	4.82	0.78	0.48	0.51
6:0	31.32	33.03	33.89	32.35	3.13	0.86	0.52	0.67
8:0	19.89	20.94	20.99	19.99	1.83	0.90	0.51	0.95
10:0	48.25	51.11	49.10	47.33	4.03	0.87	0.59	0.66
12:0	58.37	63.72	64.61	64.61	4.72	0.65	0.77	0.38
14:0	167.08 [b]	188.46 [ab]	206.56 [ab]	219.85 [a]	12.21	<0.01	0.70	<0.01
14:1	24.75 [c]	27.44 [bc]	30.23 [ab]	33.19 [a]	1.72	<0.01	0.35	<0.01
15:0	17.34	17.56	18.63	18.69	1.65	0.81	0.99	0.33
15:1	3.75	3.92	3.46	3.81	0.47	0.74	0.52	0.52
16:0	523.79	565.58	572.34	585.99	39.47	0.44	0.99	0.23
16:1 *(n9)*	7.75	7.79	7.88	7.92	0.38	0.99	0.96	0.72
16:1 *(n7)*	37.51	39.79	41.56	44.35	2.81	0.28	0.59	0.09
17:0	6.68	6.75	6.61	6.56	0.49	0.97	0.89	0.67
18:0	100.45	105.85	95.40	92.17	6.64	0.36	0.56	0.11
18:1 *(c9)*	284.60 [a]	281.86 [a]	253.52 [ab]	235.65 [b]	11.70	<0.01	0.30	<0.01
18:1 *(t9)*	20.74	19.09	18.26	19.00	1.62	0.64	0.54	0.36
18:1 *(c11)*	4.73	4.64	4.36	4.30	0.42	0.67	0.95	0.23
18:1 *(t11)*	10.11	9.83	10.24	9.71	0.78	0.92	0.63	0.99
18:2 *(t,t)*	5.36 [ab]	5.66 [a]	4.69 [ab]	4.30 [b]	0.40	0.02	0.32	<0.01
18:2 *(n6)*	32.09	36.36	30.26	29.23	3.92	0.08	0.42	0.03
18:3 *(n3)*	3.67 [a]	3.57 [a]	3.17 [ab]	2.92 [b]	0.24	<0.01	0.32	<0.01
Total 18	458.98 [a]	463.81 [a]	417.33 [b]	394.83 [b]	21.7	0.02	0.37	<0.01
19:0	2.02	2.30	1.94	2.04	0.41	0.86	0.98	0.59

20:0	2.10 [ab]	2.32 [a]	2.03 [ab]	1.53 [b]	0.21	0.03	0.04	0.02
20:1*(c8)*	2.04	1.75	1.81	1.57	0.42	0.83	0.79	0.57
20:1 *(c11)*	2.71	2.68	2.46	2.53	0.32	0.86	0.82	0.40
21:0	2.41	2.19	2.17	1.86	0.24	0.10	0.32	0.07
20:3 *(n3)*	2.21	2.26	2.09	1.91	0.19	0.55	0.46	0.21
20:4 *(n6)*	3.00	2.73	2.63	2.13	0.40	0.10	0.27	0.05
Total 20	11.25	10.96	10.28	9.02	0.77	0.09	0.24	0.03
DNFA [2]	635.49	691.43	714.70	728.06	47.65	0.31	0.92	0.12
MUFA[3]	398.37	398.50	373.48	361.76	17.13	0.25	0.61	0.05
PUFA[4]	41.15[ab]	45.08[a]	38.00[ab]	35.98[b]	0.23	0.03	0.32	<0.01
SFA[5]	1,028	1,111	1,127	1,144	73.98	0.47	0.89	0.24

[a–c] Least squares means within a row with different superscripts differ ($P < 0.05$)

[1]Fatty acid methyl ester

[2] *Denovo* FA includes C4:0, C6:0, C8:0, C10:0, C12:0, C14:0 and half of C16 yield.

[3] Mono-unsaturated FA

[4] Poly-unsaturated FA

[5] Saturated FA

Figure 3.1. Fatty acid composition of short- and medium-chain fatty acid mixture relative to *denovo* synthesized fatty acids present in milk fat

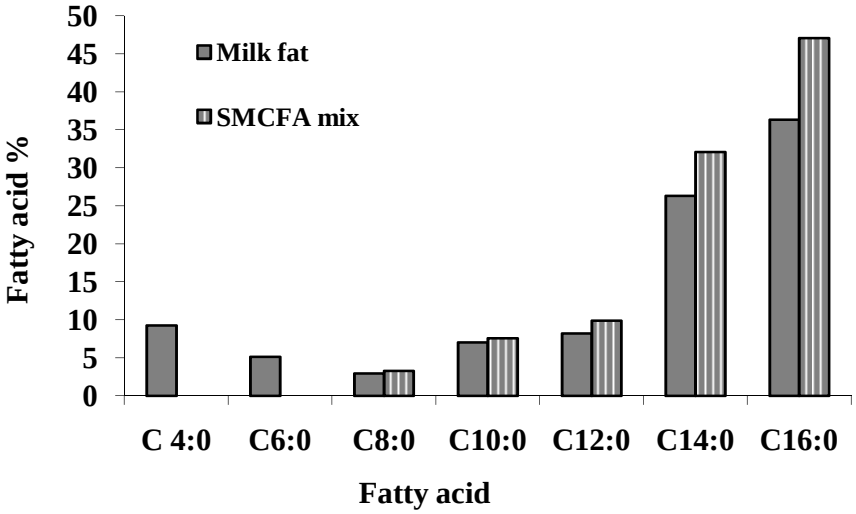
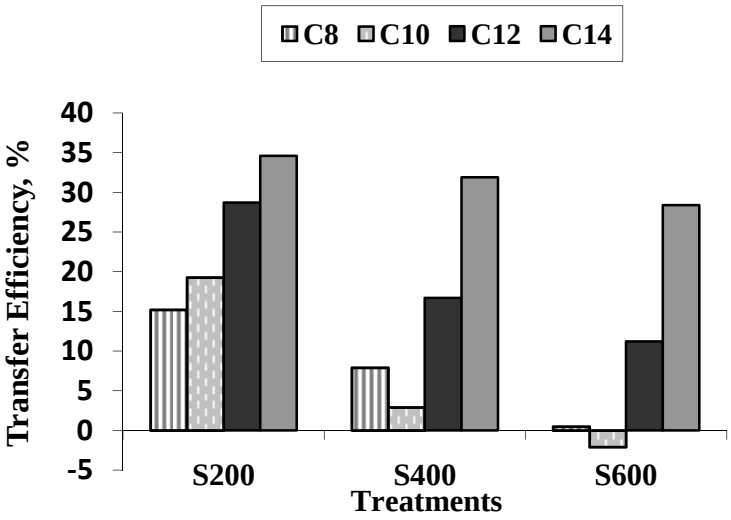


Figure 3.2. Transfer efficiency of individual short-and medium-chain fatty acids into milk fat of dairy cows fed 200, 400, and 600 g/d SMCFA



Chapter 4: EXPERIMENT 2

Milk fat responses to butterfat infusion during conjugated linoleic acid-induced milk fat depression in lactating dairy cows[1]

[1]D. Vyas, U. Moallem, B. B. Teter, P. Delmonte, R. A. Erdman. 2011. Abomasal infusion of butterfat during CLA induced milk fat depression in lactating dairy cows. J. Dairy Sci. Vol. 94, E-Suppl. 1:202.

ABSTRACT

During diet-induced milk fat depression (MFD), the short-and medium-chain fatty acids (SMCFA), which are synthesized *denovo* in the mammary gland, are reduced to a much greater extent than the long-chain fatty acids (LCFA) that originate from the circulation. Our hypothesis was that increased availability of SMCFA might rescue conjugated linoleic acid (CLA)-induced MFD in lactating dairy cows. To test that hypothesis, 4 ruminally fistulated lactating Holstein cows (128±23 DIM) were used in a 4x4 latin square design with 3 wk experimental periods. Treatments were applied during the last 2 wk of each period and included 3X daily abomasal infusion of a total of : 1) 230 g/d of LCFA (LCFA, blend of 59% cocoa butter, 36% olive oil and 5% palm oil); 2) 420 g/d butterfat (BF); 3) 230 g/d LCFA with 27 g/d CLA (LC-CLA) containing 10 g/d of *trans*-10, *cis*-12 CLA; and 4) 420 g/d butterfat with 27 g/d CLA (BF-CLA). Butterfat provided 50% of C16 and similar amounts of C-18 FA as found in LCFA such that the difference between the BF and LCFA treatments were 190g/d SMCFA. No treatment effects were observed for DMI or milk yield. Milk fat content was significantly reduced by 41% and 32%, respectively, with LC-CLA and BF-CLA. Milk fat yield was significantly reduced by 41% and 38% with LC-CLA and BF-CLA respectively compared to their respective controls. CLA infusion significantly reduced *denovo* synthesized FA (DNFA). The concentration of DNFA was significantly reduced with CLA but DNFA tended to be greater with BF infusion. Infusion of CLA significantly reduced the expression of mammary lipogenic genes involved in *denovo* FA synthesis *(ACC, FASN)*, FA uptake *(LPL)*, FA desaturation *(SCD)* and triglyceride synthesis *(AGPAT, DGAT)*. Protein abundance of the enzymes ACC and FASN that are involved in

DNFA synthesis were also significantly reduced. The increased availability of SMCFA increased *LPL* mRNA expression but no other effects were observed for lipogenic gene expression. The results suggest that nutritional manipulation by increasing the intestinal availability of SMCFA was not sufficient to rescue CLA-induced MFD.

INTRODUCTION

Milk fat synthesis depends on two general sources of FA i.e. *denovo* synthesis of FA in mammary gland and transfer of preformed FA from blood triglycerides (TG). The short-and medium-chain FA (SMCFA, C4-C14) and half of C16 are synthesized *denovo,* whereas rest FA including 50% of C16 and other long-chain FA (LCFA) are derived from TG in the blood (Jensen, 2002).

Milk fat synthesis is highly responsive to nutritional manipulation and nutrition has been used as a practical tool to alter milk fat yield and FA composition (Sutton, 1989). Certain dietary alterations including high-concentrate diets and diets high in polyunsaturated FA (PUFA) can induce a low milk fat syndrome, reducing milk fat percentage and yield up to 46% and is commonly termed as milk fat depression (MFD) (Piperova et al., 2000; Peterson et al., 2003). During MFD, mammary lipogenesis is inhibited by specific FA intermediates produced during rumen biohydrogenation (Bauman and Griinari, 2003). The relationship between *trans*-10, *cis*-12 CLA and MFD is well established (Baumgard et al., 2000). The infusion of *trans*-10, *cis*-12 CLA is accompanied with dramatic reduction of mRNA abundance of enzymes involved in *denovo* FA synthesis *(acetyl-CoA carboxylase, ACC; fatty acid synthase, FASN),* mammary uptake and intracellular transport of FA (*lipoprotein lipase, LPL; fatty acyl binding protein, FABP),* FA desaturation *(stearoyl-CoA desaturase, SCD)* and TG synthesis (*glycerol-3-phosphate acyl transferase, GPAT; acylglycerol-3-phosphate acyl transferase, AGPAT)* (Piperova et al., 2000; Peterson et al., 2003). The coordinated downregulation of mammary lipogenic enzymes during MFD suggests a major role of transcription factors such as *sterol regulatory element binding protein (SREBP-1)* in

mammary lipogenesis (Harvatine and Bauman, 2006). During diet-induced MFD, the SMCFA, which are synthesized *de novo* in the mammary gland, are reduced to a much greater extent than the long-chain fatty acids (LCFA), which originate from circulating blood TG originating from the diet and tissue-mobilized TG (Chouinard et al., 1999; Baumgard et al., 2002).

The SMCFA are essential for formation of milk TG (Moore and Steele, 1968) and for maintaining the fluidity of milk fat (Barbano and Sherbon, 1980). Recently, the importance of SMCFA during milk fat synthesis was underscored when post-ruminal infusion of butterfat, used as a source of SMCFA, increased milk fat synthesis in lactating dairy cows (Kadegowda et al., 2008). This suggests that the mammary gland is responsive to SMCFA during normal milk fat synthesis. We hypothesized that the responsiveness to SMCFA would be greater when *denovo* synthesis of these FA is inhibited. The objective of the present study was to determine whether CLA-induced MFD can be reversed/ prevented by SMCFA availability.

MATERIALS AND METHODS

Animals, Experiment Design, Treatment, and Sampling.

All procedures for this experiment were conducted under a protocol approved by the University of Maryland Institutional Animal Care and Use Committee. Four ruminally fistulated multiparous Holstein dairy cows in mid lactation (128 ± 23 DIM) were used in 4x4 Latin square design balanced for carryover effects. Treatments were abomasal infusion of the following: 1) 230 g/d of long-chain FA (LCFA); 2) 420 g/d butterfat (BF); 3) 230 g/d LCFA with 27 g/d CLA (LC-CLA) containing 10 g/d of *trans*-10, *cis*-12 CLA; and 4) 420 g/d butterfat with 27 g/d CLA (BF-CLA).

In the LCFA treatment, only 50% of the palmitic acid found in the butterfat was included, because it has been estimated that approximately 50% of palmitic acid is synthesized *de- novo* (Palmquist and Jenkins, 1980). The LCFA mixture was a blend of 59% cocoa butter (Bloomer Chocolate Company, Chicago, IL), 36% olive oil (Filippo Berio, Hackensack, NJ), and 5% palm oil (Malaysian Palm Oil Board, Washington, D.C.). In the BF treatment, butter oil was prepared from commercially available unsalted butter (Kirkland Signature, Costco Wholesale Corporation, Seattle, WA) melted at 37°C and separated from the protein coagulate by filtration. The CLA mixture was prepared from Clarinol® (Lipid Nutrition, Maywood, NJ). Amounts of postruminally infused individual FA in the LCFA mixture and butterfat are shown in Figure 4.1. The FA composition of the fat supplements is presented in Table 4.2.

Experimental periods were 3 wk. The first week of each period was without fat infusion to reduce carryover effects. This was followed by 2 wk of abomasal infusion. Fat supplements were infused via tygon tubing (0.48-cm i.d, 0.64-cm o.d; VWR Scientific, Bridgeport, NJ) that passed through the ruminal cannula, the rumen, the omasum, and into the abomasum, where the line was maintained using a 10-cm circular plastisol flange. The fat mixtures were liquified at 37°C in a warming oven and mixed well before infusion. The amount of each FA mixture was divided into equal portions and manually infused 3 times per day (140 g of butterfat, 76.6 g of LCFA, and 9 g of CLA at 0800, 1400, and 1900 h). Actual amounts of infused fat were recorded daily. Patency and location of the infusion line inside the cow were checked on alternate days.

Cows were housed in individual tie stalls equipped with rubber mats and bedded with wood shavings. Cows were fed a basal diet containing 50% forage and 50% concentrate (DM basis) to meet NRC (2001) nutrient specifications for a 600-kg cow producing 45 kg of milk containing 3.7% milk fat and 3.1% milk protein. Ingredient and chemical composition of the basal diet are given in Table 4.1. Diets were fed as TMR once daily at 0800 h. Forage and ingredient DM were measured weekly, and the TMR was adjusted accordingly to maintain a constant forage-to-concentrate ratio on a DM basis. Amounts of feed offered and refused were recorded once daily. Cows were milked twice daily at 0700 and 1700 h, and milk production was recorded electronically at each milking. Samples for milk composition and FA analysis were collected from the last 6 consecutive milkings during wk 3 of each experimental period. Milk fat, protein, and somatic cell counts (SCC) were determined by infrared analysis (Foss Milk-O-Scan, Foss Food Technology Corp., Eden Prairie, MN) on fresh samples from individual milkings. A subset of samples from each milking was composited and frozen at -20^{o}C for subsequent FA analysis. Transfer efficiency of each FA was calculated as the increased output in milk divided by the infused FA.

FA Composition Analysis

Milk FA composition was analyzed from pooled milk samples from last 6 consecutive milkings of wk 3. The FA methyl esters (FAME) were prepared by mild transesterification with 1.4 mol/L of H_2SO_4 in methanol (Christie, 1982). Separations were achieved using an Agilent 6890N gas chromatograph (Agilent Technologies., Wilmington, DE) equipped with a flame ionization detector. Hydrogen was used as carrier gas at 1 mL/min constant flow with a linear velocity of 30 cm/s. Air flow was

maintained at 400 mL/min. Nitrogen was used as make up gas a flow rate of 33 mL/min. The oven was maintained at 169^{o}C isothermal temperature, the injection port at 250^{o}C, and the detector at 250^{o}C. The split ratio was set to 1:100 and the typical injection volume was 1 uL.

The SMCFA were analyzed as fatty acid butyl esters (FABE), which were mathematically converted to FAME and normalized to the FAME chromatogram (Gander et al., 1962). The original FABE procedure was modified as follows. Milk samples were heated in screw-capped test tubes at 80^{o}C for 1 h in the presence of 1.4 N H_2SO_4 in butanol, followed by extracting with hexane in the presence of saturated KCl and distilled water. Samples were then centrifuged at 500 x *g* for 5 min at room temperature. Aliquot of the upper hexane layer was injected directly into a Hewlett-Packard 5880 gas-liquid chromatograph equipped with a split injector, a flame ionization detector and a 25 m x 0.2 mm i.d. fused silica capillary column coated with HP1 (Hewlett Packard, Avondale, PA). Helium was used as the carrier gas at a flow rate of 2 mL/min with a split ratio of 45:1. Injector temperature and detector temperature were set at 250°C while column temperature started at 130°C. Ramp was set at 6^{o}C/min to 290^{o}C, followed by 4^{o}C/min to 260^{o}C and finally holding at 260^{o}C for 20 min. Standard mixtures, including GLC-60 (Nu-check Prep, Inc., Elysian, MN), were converted to FABE to aid in the identification and quantification of FA.

Mammary Biopsy

Mammary biopsies were performed on d 21 of each experimental period. Biopsies were taken from either on the left or right rear gland. The biopsy site was carefully

selected to avoid larger subcutaneous blood vessels. Preparation of the site involved shaving and washing with dilute betadine (Purdue Frederick, Stamford, CT) solution followed by sanitizing with ethyl alcohol (70%). Cows were given intravenous xylazine before anesthetizing the biopsy site by subcutaneous injection of lidocaine hydrochloride (line block). An incision was made (~0.5-1.0 cm) on the outside of the quarter using a scalpel blade (size 22). A Bard® Magnum® core biopsy instrument (Bard Peripheral Vascular, Inc., Tempe, AZ) with a Bard® Magnum® core tissue biopsy needle (MN1210, 12G × 10 cm) was used to biopsy mammary tissue (30-50 mg tissue /biopsy). Tissue samples were snap frozen in liquid N_2 and stored at −80°C.

RNA Isolation and Quantitative Real-Time Reverse-Transcription PCR

Frozen biopsy tissues were weighed (~30 mg) and immediately subjected to RNA extraction using Qiagen RNeasy mini kit with on-column DNAse digestion (Qiagen, Valencia, CA). The RNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.9. The RNA integrity was assessed by electrophoretic analysis of 28S and 18S rRNA subunits using agarose gel electrophoresis.

A portion of the extracted RNA was diluted to 1μg/μL using DNase-RNase free water prior to reverse transcription. The cDNA was synthesized from 1μg RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA) as per the manufacturer's instructions. A negative control to check for genomic DNA contamination was prepared by pooling RNA from each sample, and using 1 μg in a

reaction without reverse transcriptase. All first-strand cDNA reactions were diluted 5-fold prior to use in PCR.

Primer sequences utilized in these experiments are detailed in Table 4.3. The mRNA levels were quantified using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and the 2X Quantitect SYBR Green PCR Master Mix (Bio-Rad). Cycles were performed as follows: denaturation at 95^{o}C for 3 min to activate the polymerase, followed by 40 cycles of 95^{o}C for 15 s, 60^{o}C for 30 s, and 72^{o}C for 30 s. The presence of a single PCR product and the absence of primer-dimers were verified by the melt curve analysis using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were normalized to the housekeeping gene *Ubiquitously expressed transcript (UXT)* after comparing the expression of *UXT, Mitochondrial ribosomal protein L39* (*MRPL39)* and *Eukaryotic translation initiation factor 3, subunit K (EIF3K)* (Kadegowda et al., 2009). The stability of housekeeping genes were tested by Normfinder software (Molecular Diagnostics Lab, Aarhus, DK). Data were transformed using equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), where Ct represents the fractional cycle number when the amount of amplified product reaches a threshold for fluorescence. The normalized data were transformed to obtain a perfect mean of 1.0 for the LCFA treatment, leaving the proportional difference between the biological replicates. The same proportional change was calculated in all the treatments to obtain a fold change relative to LCFA treatment.

Protein Isolation and Western Blotting

Protein isolation and western blotting procedures were adapted from (Rudolph et al., 2010). Briefly, mammary lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2.0 mM

EDTA, 50 mM NaF, 5.0 mM sodium vanadate, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS] to which 0.57 mM phenylmethylsulfonyl fluoride (PMSF), 20 µL/mL EDTA-free protease inhibitor cocktail (Roche, Applied Science, Indianapolis, IN), and 1.0 mM DTT were added to extract proteins from the mammary samples. Samples were homogenized using a Brinkman homogenizer, and lysate was centrifuged at 13,000 *g* for 20 min at 4°C. Protein concentrations in the supernatant were determined using bicinchoninic acid (Pierce, Rockford, IL). Proteins were resolved using 8% SDS-polyacrylamide gels(Laemmli, 1970). Resolved proteins were transferred to nitrocellulose membrane (Biorad laboratories, Hercules, CA). Antibodies directed against acetyl-CoA carboxylase-1 (Polyclonal antibodies raised in rabbit; Catalogue # 3662S) and fatty acid synthase (Polyclonal antibodies raised in rabbit; Catalogue # 3180S) and secondary antibodies (Anti-rabbit IgG, HRP linked antibody; Catalogue # 7074S) were obtained from Cell Signaling Technology (www.cellsignal.com, Danvers, MA).

Statistical Analysis

Milk production, milk components, gene expression, protein expression and FA composition data were analyzed as a 2x2 factorial arrangement of treatments in 4x4 Latin square design using the MIXED procedure in SAS (Version 9.2, SAS Institute, Cary, NC). The statistical model included the effect of cow and experimental period, type of fat (BF or LCFA), CLA (with or without CLA infusion) and fat x CLA interaction. One cow was diagnosed with displaced abomasum and surgically repaired during wk 2 of period 1. To allow for recovery from surgery, period 1 data from this cow was not included in the

analysis. A probability of $P \leq 0.05$ was considered statistically significant. The results are presented as least squares means.

RESULTS

The FA profile of the infused FA mixture is presented in Table 4.2. The CLA supplement was a mix of 42% *cis*-9, *trans*-11 and 45% *trans*-10, *cis*-12 CLA. The LCFA mixture was formulated to provide 50% of palmitic acid and the remainder of LCFA equivalent to BF treatment.

Intake and milk production responses are presented in Table 4.4. No treatment or interaction effects were observed for DMI and milk yield. However, CLA infusion reduced 3.5% FCM by 25% with BF and LCFA ($P < 0.01$). The feed conversion efficiency (FCM/DMI) was reduced with CLA ($P < 0.01$) while no effects were observed due to type of infused fat ($P = 0.46$). Milk fat percentage was reduced by 32 and 41% ($P < 0.01$) and milk fat yield was reduced by 38 and 41% ($P < 0.01$) with BF-CLA and LC-CLA respectively. There were no treatment effects on milk protein yield and content.

Concentrations and yields of individual milk FA are in Table 4.5 and 4.6, respectively. Abomasal infusion of CLA altered milk FA composition as it shifted towards higher proportions of LCFA (> 16 carbons) due to a reduction in the secretion of DNFA. Conjugated linoleic acid infusion reduced the DNFA content by 11 and 15% ($P < 0.01$) and yield by 44% and 50% ($P < 0.01$) with BF-CLA and LC-CLA, respectively. The concentration and yield of all SMCFA including C6, C8, C10 and C12, along with C16 were reduced ($P < 0.01$) with CLA infusion, with the exception of C14. The milk fat content of C14 remained constant while the yield was reduced with CLA infusion ($P <$

0.01). While milk fat content of C18:0, total C18:1 and total C18:2 were increased with CLA ($P < 0.01$) infusion, the yield of the respective FA was reduced ($P < 0.01$). Concentrations and yield of individual 18:1 and 18:2 FA isomers are in Table 4.7 and 4.8, respectively. Total *trans*-18:1 including *trans*-5/7, *trans*-8/9, *trans*-11, *trans*-13/14, and *trans*-16 increased with CLA infusion ($P < 0.01$). The concentration of total 18:1-*cis* ($P = 0.09$), *cis-9* ($P = 0.10$) and *cis*-15 ($P = 0.10$) tended to increase while *cis*-13 ($P = 0.01$) and *cis*-14 18:1 ($P = 0.04$) were increased with CLA infusion. Total CLA concentration was increased by 166 and 116% and CLA yields were increased by 56 and 43% with LC-CLA and BF-CLA, respectively ($P < 0.01$). The concentrations of individual CLA isomers including *cis*-9, *trans*-11($P < 0.01$), *trans*-10, *cis*-12 ($P < 0.01$), *trans*-9, *cis*-11($P < 0.01$) and *trans*-11, *trans*-13 ($P = 0.01$) were also increased. Yield of *cis*-9, *trans*-11 CLA tended to increase ($P = 0.06$) by 29 and 27% with LC-CLA and BF-CLA treatments, respectively, ($P < 0.01$) with a transfer efficiency of ~17%. The yield of *trans*-10, *cis*-12 was increased ($P < 0.01$) with CLA infusion with a transfer efficiency of 19 and 20% with LC-CLA and BF-CLA treatments, respectively.

The milk FA profile in response to BF or LCFA infusion without CLA was not markedly altered. Concentrations of SMCFA including C6, C8, C10, and C12 remained constant while C14:0, 14:1 *cis*-9, and 16:1 *cis*-9 increased ($P < 0.01$) with BF as compared to LCFA infusion. The total DNFA concentration tended to be higher with BF infusion ($P = 0.09$). However, no effects were observed on DNFA yield ($P = 0.43$). Similarly, the content and yield of MUFA and PUFA were similar irrespective of type of fat infused ($P = 0.72$; $P = 0.67$ respectively). The content of 18:0 was greater ($P < 0.01$)

while total 18:1 tended to be geater with LCFA infusion while the yield of the respective FA remained constant between BF and LCFA treatments.

Abomasal infusion of CLA altered mammary lipogenic enzyme and gene expression. The mRNA abundance of *ACC* and *FASN* (Figure 4.2), *AGPAT, diacylglycerol acyl transferase (DGAT)* (Figure 4.3), *LPL* (Figure 4.4), *and SREBP-1* (Figure 4.5) was reduced ($P < 0.01$) with CLA infusion, with the exception of *stearoyl-CoA desaturase (SCD)* (Figure 4.4) and *peroxisosome proliferator activated receptor-γ (PPAR-γ)* (Figure 4.5). The mRNA abundance of *LPL* was increased ($P < 0.01$) and that of *SREBP cleavage activating protein (SCAP)* (Figure 4.6) tended ($P < 0.10$) to increase with BF infusion compared to LCFA. The interaction between type of fat and CLA was significant for *SCD* ($P < 0.05$). The mRNA expression of INSIG was reduced with CLA while no effects were observed with type of fat infused (Figure 4.6). Similarly, CLA infusion reduced lipogenic protein expression in mammary gland. The expression of ACC and FASN was reduced with CLA ($P < 0.01$). However, the type of fat infused had no effect on protein expression. Similar response was observed with Fat x CLA interaction.

DISCUSSION

As SMCFA are reduced to the greater extent relative to LCFA during MFD, our hypothesis was that post-ruminal infusion of SMCFA could alleviate at least a portion of CLA-induced MFD. Butterfat, used as a source of SMCFA provided an additional 190 g/d SMCFA. Thus, compared to LCFA the responses observed by comparing BF and LCFA treatments could be attributed to SMCFA. The CLA supplement in the present

study contained two major CLA isomers, *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA. However, previous studies have shown that *cis*-9, *trans*-11 CLA isomer had no effect on milk fat synthesis in dairy cows (Baumgard et al., 2000; Baumgard et al., 2002; Loor and Herbein, 2003). Hence, it is assumed that milk fat responses with CLA supplement in the present study are due to *trans*-10, *cis*-12 CLA isomer.

Conjugated linoleic acid induced MFD in the present study further verified the role of *trans*-10, *cis*-12 CLA as a potent inhibitor of milk fat synthesis (Chouinard et al., 1999; Baumgard et al., 2000). Milk FA profile during MFD is characterized by reduced secretion of FA originating from *denovo* synthesis and reduced FA uptake from circulation with effects more pronounced on DNFA (Bauman and Griinari, 2003). The changes in milk FA profile appeared to be mainly due to reduced mammary lipogenesis which further is regulated by *SREBP-1* gene expression. The CLA-induced responses on milk FA profile and mammary lipogenic gene expression in the present study are consistent with previous studies using either pure *trans*-10, *cis*-12 CLA isomer (Baumgard et al., 2002; Gervais et al., 2009) or during diet-induced MFD (Opstvedt et al., 1967; Piperova et al., 2000; Peterson et al., 2003). However, to our knowledge, this is the first study measuring ACC and FASN protein expression during CLA-induced MFD. The protein expression of ACC and FASN reflected the changes in mRNA expression of the respective enzyme. These findings further support the assumption that mammary lipogenesis is regulated at the level of mRNA expression (Rudolph et al., 2007). In contrast, increased availability of SMCFA with BF infusion had no effects on milk fat responses unlike previous study where abomasal infusion of BF increased milk fat yield (Kadegowda et al., 2008). The differences in the responses were more apparent when the

transfer efficiency of individual SMCFA was considered. The DNFA including individual SMCFA were transferred with the greater efficiency in the previous study (Kadegowda et al., 2008). The increased availability of SMCFA along with greater mRNA abundance of both *ACC* and *FASN* might explain greater milk fat responses with BF infusion (Kadegowda, 2008). It is difficult to reconcile the differences between both studies. However, the cows used in the present study had higher average milk production (48 vs. 32 kg/d) that might have affected the milk fat responses. The higher energy requirements for lactation might have reduced the transfer efficiency of SMCFA by increasing its extra-mammary utilization. Short-chain FA can be absorbed directly from the digestive tract into the portal vein and can be preferentially oxidized in liver as energy substrates (Souza and Williamson, 1993).

The mRNA expression of *LPL* was reduced in cows receiving *trans*-10, *cis*-12 CLA (Baumgard et al., 2002; Harvatine and Bauman, 2006; Gervais et al., 2009). Consistent with the previous findings, the mRNA abundance of *LPL* was reduced with CLA infusion in the present study. Lipoprotein lipase plays an important role in hydrolysis of plasma TG and is important for delivery of dietary FA to the mammary gland (Fielding and Frayn, 1998). The CLA-induced downregulation of *LPL* further suggests reduced uptake of blood TG. However, greater substrate availability with BF infusion increased *LPL* expression further increasing the availability of LCFA for their utilization by mammary gland (Annison et al., 1968).

The improved milk fat responses with BF infusion was previously (Kadegowda, 2008) attributed to the increased expression of *LPL* along with enzymes involved in TG

synthesis; *AGPAT* and *DGAT*. However, the expression of *DGAT* was not affected in the present study. Diacylglycerol acyl trasferase catalyzes the committed and final step of TG synthesis determining the flux of available substrates into TG (Yen et al., 2008). This might explain the lack of milk fat response with BF infusion despite increased substrate availability.

Stearoyl-CoA desaturase-1 regulates the availability of 18:1*c9* by introducing a *cis-9* double bond on the saturated chain of C18 (Cook et al., 1976). However, its specificity extends to other MCFA or LCFA such as C14 and C16 (Ntambi et al., 2004). The desaturase index measured as product: substrate ratio has been used as indirect measure for *SCD* activity (Table 4.9). *Trans*-10, *cis*-12 CLA reduced desaturase index in some experiments (Baumgard et al., 2002) while no effects were observed in others (Gervais et al., 2009). Similarly, *trans*-10, *cis*-12 CLA can reduce mRNA expression by downregulating the transcriptional enhancer element of the *SCD-1* gene promoter (Keating et al., 2006). However, the increased availability of SMCFA had no effect on either desaturase index or mRNA expression (Kadegowda et al., 2008). In the present study, both CLA and fat source had no effect on desaturase index reflecting the lack of change in *SCD-1* mRNA expression due to respective treatment. For unknown reasons, there was a FAT x CLA interaction for *SCD-1* gene expression. However, the effects were not reflected on desaturase index. The lack of correlation between desaturase index and mRNA expression has been observed previously in bovine mammary gland (Bionaz and Loor, 2008) and could be attributed to posttranslational modifications, and factors affecting the synthesis and secretion of FA related to *SCD-1* activity (Gervais et al., 2009).

Although the molecular mechanisms regulating milk fat synthesis are not well established, the coordinated down-regulation of mammary lipogenesis suggests the involvement of transcription regulation (Peterson et al., 2004; Harvatine and Bauman, 2006). The role of *SREBP-1* has been suggested as a global regulator of mammary lipid metabolism (Rudolph et al., 2007) both *invitro* in mammary epithelial cells (Peterson et al., 2004) and in lactating dairy cows (Harvatine and Bauman, 2006). The transcription factor *SREBP-1* is synthesized as precursor protein associated with *SCAP* and is anchored to the endoplasmic reticulum with *INSIG-1* protein. Upon activation, the *SREBP* precursor undergoes a sequential two step cleavage process and the mature protein is translocated to the nucleus where it binds the target genes on sterol response elements (Wang et al., 1994; Sakai et al., 1998). *Trans*-10, *cis*-12 CLA downregulates the nuclear abundance of *SREBP-1* by inhibiting proteolytic activation process of *SREBP*-1 protein or by inhibiting *SREBP-1* gene transcription (Bernard et al., 2008). The mRNA abundance of its active nuclear fragment was decreased in response to *trans*-10, *cis*-12 CLA in dairy cows (Harvatine and Bauman, 2006; Gervais et al., 2009), mice (Kadegowda et al., 2010) and bovine mammary epithelial cells (Peterson et al., 2004). The mRNA expression of *SCAP* and *INSIG-1* were reduced in response to *trans*-10, *cis*-12 CLA, suggesting post-transcriptional secondary regulation of *SREBP-1* (Harvatine and Bauman, 2006). The CLA effects in the present study agree with the previous findings. However, increased availability of SMCFA had no effects on *SREBP-1* expression contrary to what was observed earlier (Kadegowda, 2008).

The *PPAR-γ* is an important member of the nuclear receptor super family of transcription factors. Bionaz and Loor (2008) suggested that *PPAR- γ* could be the main

transcription factor controlling milk fat synthesis by serving as a regulator for *SREBP* activity. Kadegowda et al. (2009) also showed marked upregulation of mammary lipogenic gene expression with Rosiglitazone, *PPAR-γ* agonist, in bovine mammary epithelial cells. However, the mRNA expression of *PPAR-γ* was not altered in the present study which agrees with the hypothesis that *SREBP-1*as the major regulator of mammary lipogenesis (Harvatine and Bauman, 2006).

CONCLUSION

While CLA reduced mammary lipogenesis, the increased availability of SMCFA failed to rescue CLA-induced MFD. The milk fat responses with SMCFA were small and non-significant with no effects observed on either mRNA or protein expression of lipogenic enzymes. The results suggest that nutritional manipulation with intestinal SMCFA was insufficient to rescue CLA-induced MFD. This suggests that CLA-induced MFD is caused by more than just an insufficient supply of SMCFA, typically provided by *denovo* FA synthesis in the mammary gland.

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Table 4.1. Ingredient and chemical composition of the basal diet

Item	DM %
Ingredient	
Corn silage	50.02
Corn grain, Ground	26.01
Soybean meal	20.21
Corn gluten meal (60%)	0.45
Limestone	0.62
Calcium Phosphate	0.43
Magnesium oxide	0.16
Sodium bicarbonate	0.57
Dynamate	0.13
Salt	0.38
Trace minerals and Vitamins	0.46
Megalac	0.56
Chemical composition	
DM, %	60.88
CP, %	16.69
RUP, %[1]	43.02
ADF, %	16.98
NDF, %	29.21
NE_L, Mcal/kg[2]	1.56
Ca	0.81
P	0.45
Mg	0.32
K	1.19
Na	0.35
Cl	0.41

[1]Trace mineral and vitamin mix combined which provided an additional; 0.76 mg/kg Co, 10 mg/kg Cu, 5.5 mg/kg Fe, 0.64mg/kg I, 37 mg/kg Zn, .33 mg/kg Se, 3,526 IU/kg vitamin A, 1175 IU/kg vitamin D, and 22 IU/kg vitamin E to the diet DM.
[2]Calculated value based the estimated TDN from feed analysis at 3X maintenance intake using NRC (2001) prediction equations.

Table 4.2. Fatty acid composition of fat supplements infused in lactating dairy cows

	CLA	Butterfat	Palm oil	Olive oil	Cocoa Butter	LCFA
			g/100g FAME[1]			
4:0		4.52				
6:0		2.40				
8:0		1.36				
10:0		2.94				
12:0		3.39	0.25			0.01
13:0		0.16				
14:0	0.03	9.87	1.06	0.03	0.11	0.13
15:0		1.48	0.05		0.03	0.02
14:1		0.73				
16:0	0.18	27.46	41.15	13.82	25.25	22.06
16:1	0.07	1.23	0.16	1.24	0.24	0.60
17:0		1.14	0.10	0.08	0.23	0.17
18:0	0.07	11.85	4.16	2.65	36.40	22.59
17:1		0.19	0.03	0.12		0.04
18:1 *cis*-9	5.26	21.12	40.82	69.12	32.83	46.41
18:1 *cis*-11	0.37	0.53	0.75	2.78	0.34	1.24
18:1 *cis*-12		0.51	0.00			
18:1 *cis*-13	0.11	0.06	0.01			
18:1 *cis*-14		0.05				
18:1 *trans* total		3.52				
18:2 *trans*-11,*cis*-15	0.14	0.07	0.19	0.03		0.02
18:2 *cis*-9, *cis*-12	0.79	2.98	10.26	8.61	2.91	5.26
18:3		0.34	0.18	0.42	0.17	0.26
20:0	0.15	0.16	0.42	0.49	1.15	0.81
20:1 *cis*-9		0.10	0.01	0.00		
20:1 *cis*-11		0.04	0.15	0.30	0.05	0.15
20:2	1.18					
22:0		0.04	0.08	0.16	0.21	0.19
18:2 *trans*-7,*cis*-9		0.04				
18:2, *cis*-9,*trans*-11	42.55	0.47				
18:2, *cis*-10,*trans*-12	0.89					
18:2, *trans*-9,*cis*-11	0.89					
18:2, *cis*-11,*trans*-13	0.73					
18:2, *trans*-10,*cis*-12	45.56					
C20:3		0.11				
C20:4		0.16				

[1] Fatty acid methyl esters

Table 4.3. Features of primers used for qPCR analysis

Gene	Accession #		Primers (5'-3')[1]	bp[2]	Reference
ACACA	AJ132890	F	CATCTTGTCCGAAACGTCGAT	102	Bionaz and Loor, 2008a
		R	CCCTTCGAACATACACCTCCA		
AGPAT6	DY208485	F	AAGCAAGTTGCCCATCCTCA	101	Bionaz and Loor, 2008b
		R	AAACTGTGGCTCCAATTTCGA		
DGAT1	NM_174693	F	CCACTGGGACCTGAGGTGTC	101	Bionaz and Loor, 2008a
		R	GCATCACCACACACCAATTCA		
FASN	CR552737	F	ACCTCGTGAAGGCTGTGACTCA	92	Bionaz and Loor, 2008a
		R	TGAGTCGAGGCCAAGGTCTGAA		
INSIG1	XM_614207	F	AAAGTTAGCAGTCGCGTCGTC	120	Bionaz and Loor, 2008a
		R	TTGTGTGGCTCTCCAAGGTGA		
SCD-1	AY241933	F	TCCTGTTGTTGTGCTTCATCC	101	Bionaz and Loor, 2008a
		R	GGCATAACGGAATAAGGTGGC		
LPL	BC118091	F	ACACAGCTGAGGACACTTGCC	101	Bionaz and Loor, 2008a
		R	GCCATGGATCACCACAAAGG		
PPAR-γ	NM_181024	F	CCAAATATCGGTGGGAGTCG	101	Bionaz and Loor, 2008a
		R	ACAGCGAAGGGCTCACTCTC		
SCAP	DV935188	F	CCATGTGCACTTCAAGGAGGA	108	Harvatine and Bauman, 2006
		R	ATGTCGATCTTGCGTGTGGAG		
SREBF1	DV921555	F	CCAGCTGACAGCTCCATTGA	67	Loor et al., 2005
		R	TGCGCGCCACAAGGA		
UXT	NM_001037471	F	CAGCTGGCCAAATACCTTCAA	125	Kadegowda et al., 2009
		R	GTGTCTGGGACCACTGTGTCAA		
MRPL39	NM017446	F	AGGTTCTCTTTTGTTGGCATCC	101	Kadegowda et al., 2009
		R	TTGGTCAGAGCCCCAGAAGT		
EIF3K	NM_001034489	F	CCAGGCCCACCAAGAAGAA	125	Kadegowda et al., 2009
		R	TTATACCTTCCAGGAGGTCCATGT		

[1] Primer direction (F – forward; R – reverse)
[2] Amplicon size in base pairs (bp)

Table 4.4. Production responses from cows abomasally infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

	Treatments					*P*-value		
Item	LCFA	LC-CLA	BF	BF-CLA	SEM	Fat	CLA	Fat x CLA
DMI, kg/d	25.4	22.6	24.9	24.0	1.15	0.66	0.12	0.41
Milk, kg/d	47.9	46.9	49.2	46.1	5.77	0.91	0.21	0.49
3.5% FCM, kg/d	46.7	35.5	47.9	35.9	4.97	0.54	<0.01	0.13
3.5% FCM/DMI	1.84	1.69	1.93	1.48	0.23	0.46	<0.01	0.09
Milk fat, %	3.36	1.99	3.37	2.30	0.19	0.41	<0.01	0.46
Milk fat, g/d	1,600	937	1,640	1,013	163.91	0.50	<0.01	0.83
Milk protein, %	2.93	2.97	2.98	3.05	0.06	0.33	0.40	0.87
Milk protein, g/d	1,410	1,394	1,471	1,388	180.03	0.59	0.36	0.53
MUN, mg/dL	14.3	12.0	12.5	11.9	1.31	0.40	0.21	0.46

Table 4.5. Least squares means for fatty acid composition of milk from cows infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

Fatty acid	Treatments				SEM	*P*-values[1]		
	LCFA	LC- CLA	BF	BF- CLA		Fat	CLA	Fat x CLA
	g/100 g of FAME[2]							
4:0	3.59	3.44	3.28	3.15	0.58	0.52	0.12	0.98
6:0	2.12	1.47	1.91	1.43	0.19	0.35	<0.01	0.52
8:0	1.29	0.91	1.18	0.87	0.10	0.33	<0.01	0.66
10:0	2.96	2.08	2.86	1.96	0.25	0.42	<0.01	0.95
12:0	3.52	2.72	3.73	2.78	0.28	0.35	0.01	0.60
13:0	0.22	0.15	0.28	0.17	0.03	0.08	<0.01	0.40
14:0	11.09	10.78	12.19	11.51	0.38	<0.01	0.09	0.41
14:1(*c9)*	1.01	1.09	1.33	1.25	0.15	0.01	0.98	0.25
15:0	1.06	0.91	1.39	1.01	0.17	0.11	0.06	0.38
16:0	30.33	27.62	31.18	28.97	0.75	0.13	0.01	0.70
16:1 (*c9)*	1.51	1.56	1.82	1.76	0.19	<0.01	0.96	0.41
16:1 (*c11)*	0.04	0.07	0.06	0.09	0.01	0.01	<0.01	0.54
17:0	0.47	0.48	0.52	0.48	0.04	0.09	0.36	0.18
18:0	7.76	9.23	6.75	8.68	0.71	<0.01	<0.01	0.18
18:1	24.38	27.02	22.39	25.28	1.51	0.07	0.02	0.89
18:2	3.85	5.26	3.76	4.78	0.41	0.47	0.02	0.62
18:3 *(c9,c12,c15)*	0.24	0.26	0.25	0.26	0.02	0.89	0.52	0.79
20:0	0.14	0.15	0.12	0.15	0.00	0.10	0.01	0.34
20:1 *(c9)*	0.09	0.09	0.09	0.09	0.00	0.69	0.22	0.83
22:0	0.02	0.02	0.01	0.02	0.00	0.05	0.49	0.20
22:4	0.04	0.03	0.04	0.03	0.00	0.89	0.04	0.66

22:5	0.05	0.04	0.05	0.04	0.00	0.91	<0.01	0.55
Other	4.18	4.56	4.82	4.79	0.17	0.02	0.22	0.17
DNFA[3]	42.57	36.06	43.27	38.64	1.52	0.09	<0.01	0.28
MUFA[4]	27.69	30.63	26.51	29.39	1.59	0.19	0.01	0.97
PUFA[5]	4.34	5.78	4.26	5.33	0.44	0.54	0.02	0.65
SFA[6]	66.27	61.62	67.18	63.29	2.15	0.32	0.01	0.76

[1] Probability that the FAT, CLA or interaction effects were not different from zero
[2] Fatty acid methyl esters
[3] *Denovo* synthesized fatty acids
[4] Monounsaturated fatty acids
[5] Polyunsaturated fatty acids
[6] Saturated fatty acids

Table 4.6. Least squares means for fatty acid yield of milk from cows abomasally infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

Fatty acid	Treatments				SEM	*P*-values[1]		
	LCFA	LC- CLA	BF	BF- CLA		Fat	CLA	Fat x CLA
	g of FAME[2] per day							
4:0	56.5	33.9	52.3	37.0	8.84	0.94	0.03	0.60
6:0	33.7	14.5	31.4	14.8	3.95	0.71	<0.01	0.63
8:0	20.7	8.8	19.6	8.9	2.72	0.77	<0.01	0.75
10:0	48.2	20.3	47.6	20.1	7.14	0.93	<0.01	0.96
12:0	57.6	26.3	62.2	28.0	8.99	0.56	<0.01	0.79
13:0	3.5	1.4	4.7	1.5	0.73	0.29	<0.01	0.33
14:0	178.8	102.9	200.7	116.0	21.44	0.13	<0.01	0.67
14:1(*c9)*	15.8	10.0	21.5	12.7	2.22	<0.01	<0.01	0.17
15:0	17.6	8.7	23.5	9.1	3.99	0.30	<0.01	0.35
16:0	489.4	261.0	512.1	291.0	57.00	0.39	<0.01	0.90
16:1 (*c9)*	24.2	14.6	30.0	17.6	3.94	<0.01	<0.01	0.21
16:1 (*c11)*	0.6	0.7	1.0	0.9	0.14	<0.01	0.40	0.13
17:0	7.6	4.5	8.8	4.8	1.19	0.29	<0.01	0.49
18:0	123.3	85.9	110.8	94.83	15.41	0.87	0.06	0.36
18:1	384.5	246.9	365.8	255.7	32.39	0.75	<0.01	0.39
18:2	61.0	48.0	61.4	49.2	5.99	0.81	<0.01	0.89
18:3 *(c9,c12,c15)*	3.8	2.4	4.1	2.7	0.31	0.14	<0.01	0.98
20:0	2.2	1.4	1.2	1.5	0.25	0.81	0.02	0.38
20:1 *(c-9)*	1.3	0.8	1.4	0.9	0.14	0.33	<0.01	0.76
22:0	0.4	0.2	0.3	0.2	0.06	0.60	0.03	0.70
22:4	0.7	0.3	0.7	0.3	0.12	0.75	<0.01	0.61

22:5	0.9	0.4	0.9	0.4	0.13	0.74	<0.01	0.86
DNFA[3]	685.7	343.8	710.3	395.9	82.54	0.43	<0.01	0.77
MUFA[4]	437.1	280.3	433.1	295.7	37.85	0.72	<0.01	0.54
PUFA[5]	68.5	52.9	69.8	54.7	6.75	0.67	<0.01	0.94
SFA[6]	1066.9	585.4	1105.0	646.5	127.60	0.51	<0.01	0.88

[1] Probability that the FAT, CLA or interaction effects were not different from zero
[2] Fatty acid methyl esters
[3] *Denovo* synthesized fatty acids
[4] Monounsaturated fatty acids
[5] Polyunsaturated fatty acids
[6] Saturated fatty acids

Table 4.7. Least squares means for fatty acid composition of C18:1 and C18:2 isomers in milk fat from cows abomasally infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

Fatty acid	Treatments				SEM	*P*-values[1]		
	LCFA	LC-CLA	BF	BF-CLA		Fat	CLA	Fat x CLA
	g/100 g of FAME[2]							
18:1								
trans-5/7	0.13	0.20	0.13	0.22	0.02	0.22	<0.01	0.15
trans-8/9	0.28	0.37	0.36	0.40	0.04	0.03	0.02	0.20
trans-10	0.37	1.01	0.83	1.08	0.43	0.37	0.17	0.49
trans-11	0.75	0.92	0.65	0.91	0.11	0.54	0.04	0.61
trans-12	0.35	0.30	0.34	0.36	0.07	0.66	0.76	0.57
trans-13/14	0.40	0.56	0.55	0.65	0.05	<0.01	<0.01	0.11
trans-16	0.19	0.22	0.21	0.26	0.01	<0.01	<0.01	0.32
Total *trans*	2.44	3.57	3.08	3.82	0.53	0.16	0.01	0.49
cis-9	20.94	22.35	18.23	20.35	1.79	0.05	0.10	0.70
cis-11	0.63	0.71	0.63	0.69	0.07	0.80	0.15	0.70
cis-12	0.22	0.21	0.25	0.27	0.03	0.11	0.82	0.62
cis-13	0.04	0.05	0.05	0.07	0.01	0.02	0.01	0.59
cis-14	0.04	0.03	0.05	0.04	0.00	<0.01	0.04	0.50
cis-15	0.05	0.07	0.07	0.08	0.01	0.06	0.10	0.30
cis-16	0.01	0.01	0.01	0.02	0.00	0.11	0.39	0.43
Total *cis*	21.92	23.44	19.30	21.49	1.79	0.05	0.09	0.72
18:2								
trans-11, trans-15	0.02	0.02	0.02	0.02	0.00	0.04	0.69	0.87
trans 9, trans-12	0.09	0.07	0.10	0.09	0.01	<0.01	0.03	0.49

cis-9, trans-13	0.09	0.10	0.15	0.13	0.01	<0.01	0.22	0.04
trans-11, cis-15	0.04	0.05	0.05	0.05	0.00	0.99	0.44	0.75
cis-9, cis-12	3.10	3.66	2.85	3.23	0.30	0.26	0.14	0.74
cis-9, cis-15	0.02	0.04	0.03	0.03	0.00	0.86	0.37	0.18
CLA								
cis-9, trans-11	0.39	0.87	0.44	0.83	0.09	0.99	<0.01	0.60
trans-10, cis-12	0.01	0.27	0.01	0.25	0.04	0.77	<0.01	0.71
trans-7, cis-9	0.04	0.04	0.05	0.04	0.00	0.08	0.60	0.22
trans-8, cis-10	0.03	0.04	0.03	0.01	0.00	0.13	0.99	0.16
trans-9, cis-11	0.01	0.03	0.01	0.02	0.00	0.83	<0.01	0.06
trans-11,trans-13	0.01	0.03	0.01	0.02	0.00	0.26	0.01	0.25
Total CLA	0.50	1.33	0.56	1.21	0.12	0.81	<0.01	0.47

[1] Probability that the FAT, CLA or interaction effects were not different from zero
[2]Fatty acid methyl esters

Table 4.8. Least squares means for fatty acid yield of C18:1 and C18:2 isomers in milk fat from cows abomasally infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

Fatty acid	Treatments				SEM	*P*-values[1]		
	LCFA	LC-CLA	BF	BF-CLA		Fat	CLA	Fat x CLA
	g of FAME[2] per day							
18:1								
trans-5/7	2.08	1.90	2.12	2.38	0.33	0.07	0.72	0.11
trans-8/9	4.50	3.59	5.99	4.16	0.87	0.02	<0.01	0.22
trans-10	5.67	10.69	14.14	13.25	5.83	0.13	0.53	0.38
trans-11	11.64	8.81	10.69	10.05	1.63	0.88	0.13	0.30
trans-12	5.61	2.93	5.61	3.81	1.17	0.64	0.06	0.65
trans-13/14	6.48	5.42	9.13	6.88	1.38	0.01	0.03	0.34
trans-16	3.01	2.08	3.49	2.68	0.41	0.07	0.01	0.81
Total *trans*	39.00	35.43	51.18	41.51	9.83	0.10	0.21	0.54
cis-9	329.54	201.04	296.56	202.85	24.14	0.23	<0.01	0.19
cis-11	10.10	6.70	10.65	6.53	1.58	0.79	<0.01	0.61
cis-12	3.52	1.99	4.12	2.69	0.43	0.04	<0.01	0.86
cis-13	0.59	0.51	0.86	0.75	0.16	0.01	0.23	0.80
cis-14	0.58	0.32	0.75	0.40	0.11	<0.01	<0.01	0.14
cis-15	0.76	0.69	1.18	0.85	0.24	0.03	0.11	0.25
cis-16	0.18	0.11	0.24	0.18	0.05	0.20	0.18	0.86
Total *cis*	345.26	211.37	314.36	213.99	26.03	0.28	<0.01	0.21
18:2								
trans-11, trans-15	0.31	0.18	0.39	0.23	0.06	0.08	<0.01	0.63
trans 9, trans-12	1.40	0.69	1.74	0.97	0.25	0.07	<0.01	0.84

cis-9, trans-13	1.52	0.99	2.5	1.33	0.32	<0.01	<0.01	<0.01
trans-11, cis-15	0.69	0.48	0.75	0.52	0.09	0.44	0.02	0.89
cis-9, cis-12	49.02	33.32	46.47	33.19	4.36	0.55	<0.01	0.59
cis-9, cis-15	0.37	0.34	0.46	0.30	0.08	0.66	0.08	0.18
CLA								
cis-9, trans-11	6.12	7.94	7.13	9.04	0.94	0.22	0.06	0.95
trans-10, cis-12	0.11	2.41	0.17	2.61	0.30	0.61	<0.01	0.77
trans-7, cis-9	0.60	0.41	0.86	0.47	0.13	0.03	<0.01	0.09
trans-8, cis-10	0.46	0.36	0.45	0.19	0.11	0.32	0.09	0.38
trans-9, cis-11	0.16	0.26	0.24	0.25	0.06	0.24	0.11	0.15
trans-11,trans-13	0.19	0.25	0.26	0.22	0.04	0.54	0.69	0.16
Total CLA	7.74	12.06	9.07	12.95	1.25	0.32	<0.01	0.83

[1] Probability that the FAT, CLA or interaction effects were not different from zero
[2]Fatty acid methyl esters

Table 4.9. Least squares means of desaturase indices in milk from cows abomasally infused with long-chain FA (LCFA), LCFA with conjugated linoleic acid (LC-CLA), butterfat (BF) and BF with CLA (BF-CLA)

SCD1 index[2]	Treatments				SEM	*P*-value[1]		
	LCFA	LC-CLA	BF	BF-CLA		Fat	CLA	Fat x CLA
cis-9 14:1	0.083	0.093	0.098	0.099	0.012	0.02	0.13	0.22
cis-9 16:1	0.047	0.053	0.055	0.057	0.005	<0.01	0.04	0.19
cis-9 18:1	0.730	0.703	0.728	0.694	0.025	0.68	0.05	0.81
Overall index[3]	0.323	0.342	0.299	0.320	0.021	0.08	0.120	0.94

[1]Probability that the FAT, CLA or interaction effects were not different from zero

[2]Specific ratios for *stearoyl-coenzyme A desaturase-1* (*SCD1*) activity:*cis*-9 14:1 = (*cis*-9 14:1)/(*cis*-9 14:1 + 14:0); *cis*-9 16:1 = (*cis*-9 16:1)/(*cis*-9 16:1 + 16:0); *cis*-9 18:1 = (*cis*-9 18:1)/(*cis*-9 18:1 + 18:0)

[3]Overall SCD1 index was calculated as follows: ([cis-9 14:1] + [*cis*-9 16:1] + [*cis*-9 18:1])/ ([*cis*-9 14:1 + 14:0] + [*cis*-9 16:1 + 16:0] + [*cis*-9 18:1 + 18:0]).

Figure 4.1. Fatty acid composition of abomasally infused butterfat and long-chain fatty acids

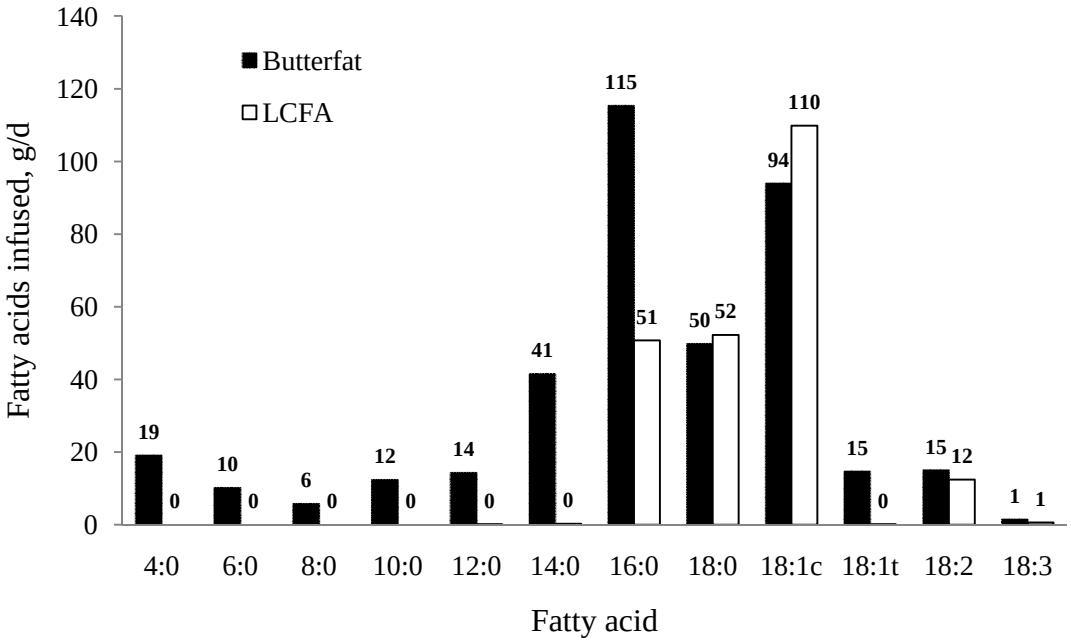


Figure 4.2. Relative mRNA abundance *of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN)* in response to abomasal infusion of different fat supplements (Bars represent the least squares means ± SEM of the respective gene); n-3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA).

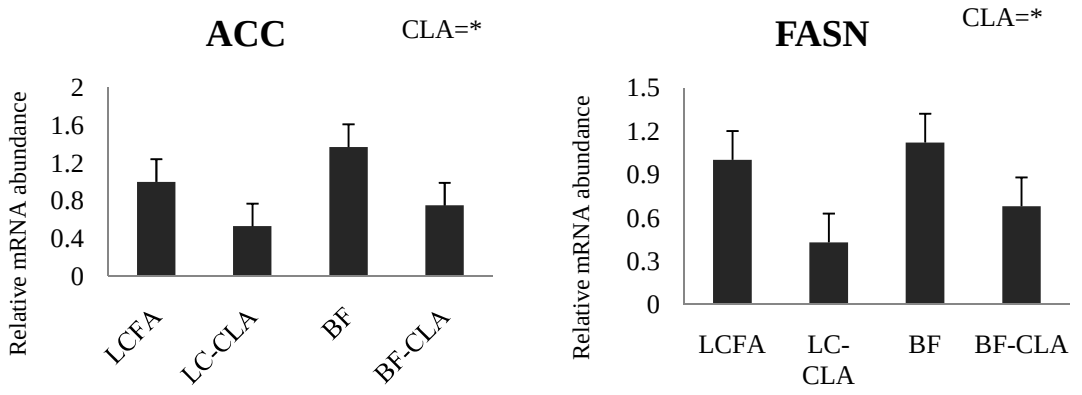


Figure 4.3. Relative mRNA abundance of *acylglycerol-3-phosphate acyl transferase (AGPAT) and diacylglycerol acyl transferase (DGAT)* in response to abomasal infusion of various fat supplements (Bars represent the least squares means ± SEM of the respective gene); n-3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA).

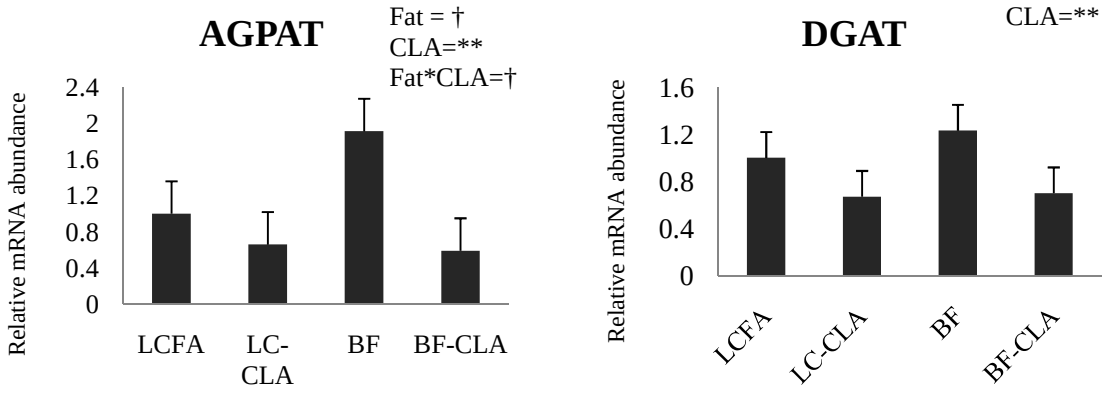


Figure 4.4. Relative mRNA abundance of *lipoprotein lipase (LPL) and stearoyl-CoA desaturase (SCD)* in response to abomasal infusion of various fat supplements (Bars represent the least squares means ± SEM of the respective gene); n-3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA)

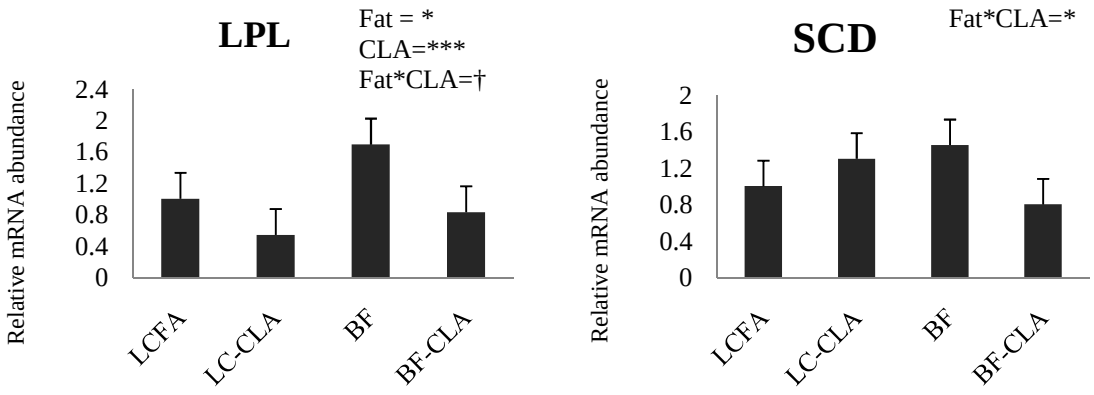


Figure 4.5. Relative mRNA abundance of *sterol regulatory element binding protein (SREBP)* and *peroxisome proliferator activated receptor (PPAR-γ)* in response to abomasal infusion of various fat supplements (Bars represent the least squares means ± SEM of the respective gene); n-3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA)

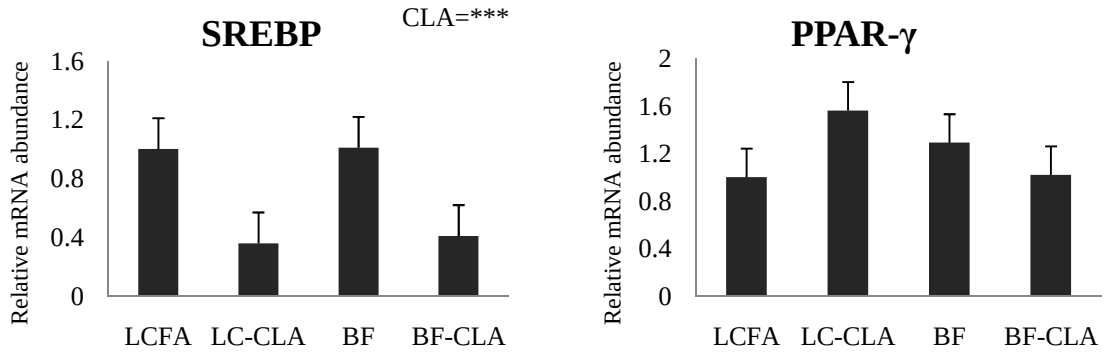


Figure 4.6. Relative mRNA abundance of *SREBP cleavage activating protein (SCAP)* and *insulin induced gene 1 protein (INSIG-1)* in response to abomasal infusion of various fat supplements (Bars represent the least squares means ± SEM of the respective gene); n-3 or 4(† $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA)

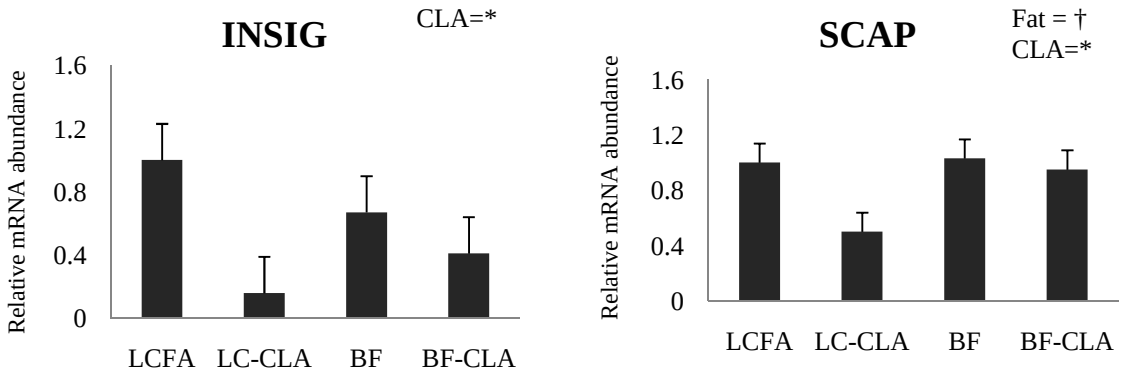


Figure 4.7. Immunoblots for acetyl-CoA carboxylase (ACC-α) protein expression in mammary tissue in response to abomasal infusion of different fat supplements (Bars represent the least squares means ± SEM of the ACC-α (normalized to *β-tubulin*); n=3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA)

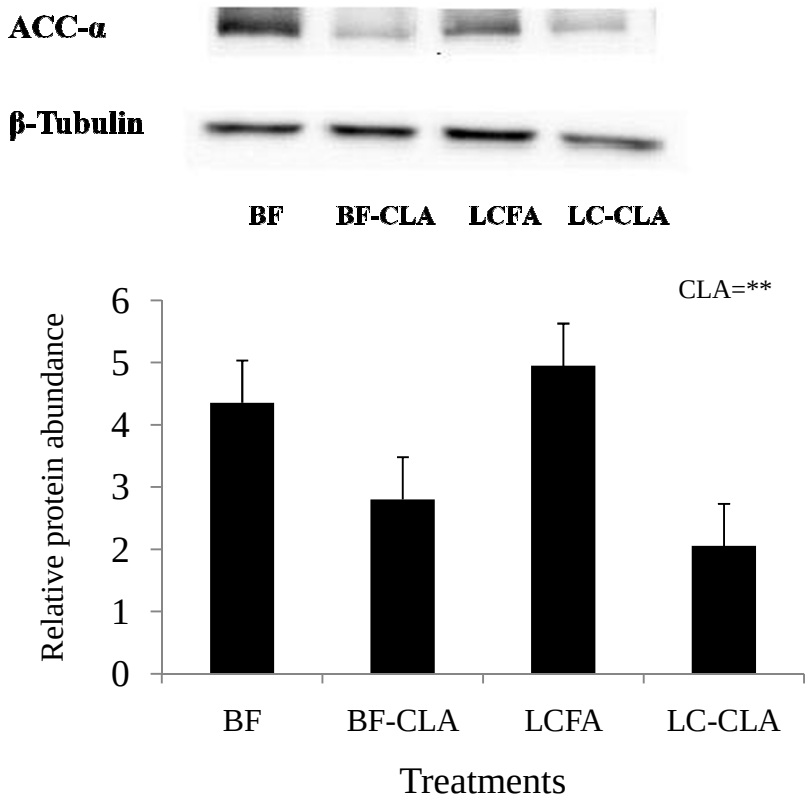
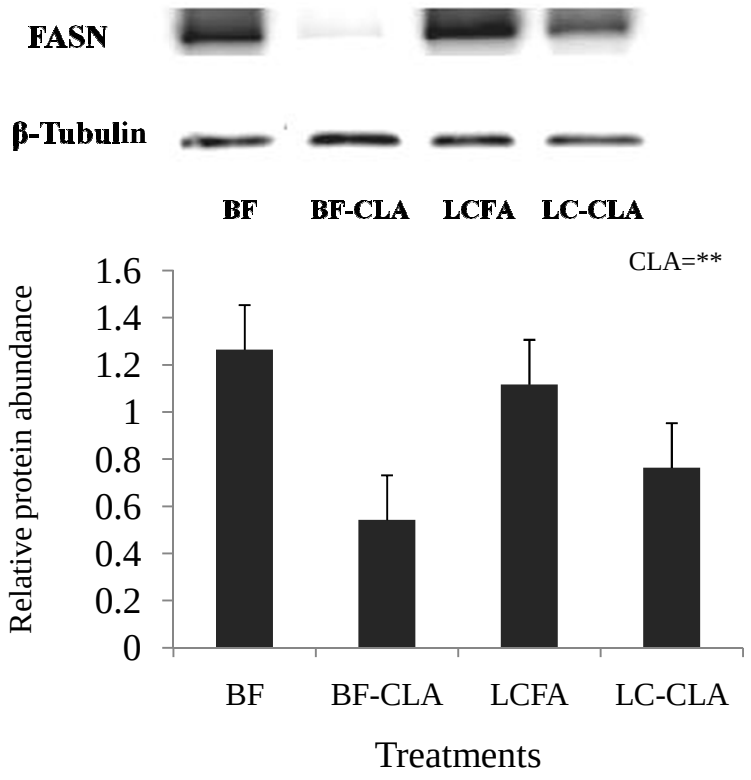


Figure 4.8. Immunoblots for fatty acid synthase (FASN) protein expression in mammary tissue in response to abomasal infusion of different fat supplements (Bars represent the least squares means ± SEM of the FASN (normalized to *β-tubulin*); n=3 or 4 († $P < 0.10$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$); LCFA(long-chain fatty acid), LC-CLA (LCFA with conjugated linoleic acid), BF (Butterfat), BF-CLA (BF with CLA)



Chapter 5: EXPERIMENT 3

Rosiglitazone corrects conjugated linoleic acid induced hepatic steatosis but not milk fat depression in lactating mice[1]

[1]D. Vyas, B. B. Teter, and R. A. Erdman. 2011. The effects of *PPAR-γ* agonist and conjugated linoleic acid on mammary and hepatic lipid metabolism in lactating mice. J. Dairy Sci. Vol. 94, E-Suppl. 1:207.

ABSTRACT

Previous studies have demonstrated the antagonizing effects of *PPAR*-γ agonists on conjugated linoleic acid (CLA)-induced hepatic steatosis and adipose tissue lipodystrophy in mice. We hypothesized that the *PPAR*-γ agonist, Rosiglitazone (ROSI), might also antagonize the CLA-induced reduction in milk fat synthesis in lactating mice. Our objective was to investigate the combination of ROSI and CLA on mammary and hepatic lipogenesis in lactating C57Bl/6J mice. Twenty-four lactating mice were randomly assigned to one of four treatments applied from Day 6 to 10 postpartum. Treatments included: 1) Control diet; 2) Control plus 1.5 % dietary CLA (CLA); 3) Control plus intra-peritoneal (IP) ROSI injections (10 mg/kg BW) (ROSI); and 4) CLA plus ROSI (CLA-ROSI). Dam food intake was significantly reduced with CLA while not with ROSI. Milk fat concentration was depressed significantly (42%) by CLA but no effects were observed with ROSI or ROSI x CLA interaction. The milk fat responses from CLA were reflected in mammary lipogenic gene and protein expression. While CLA significantly reduced mammary lipogenesis including *denovo* FA synthesis, uptake, and desaturation, and TG synthesis, no effects were observed with ROSI. Liver weight (g/100g body weight) was significantly increased by CLA due to an increase in lipid accumulation. However, ROSI rescued CLA-induced hepatic steatosis. Increased hepatic lipid accumulation with CLA triggered a compensatory reduction in mRNA abundance of hepatic lipogenic enzymes including *ACC* and *SCD-1*. However, the combination of ROSI and CLA reduced *FASN* and tended to reduce *ACC* and *LPL* mRNA expression. Pup weight gain was significantly reduced with CLA and to a lesser extent by ROSI. Overall, ROSI corrected the apparent steatosis effect of CLA but was not able to rescue

CLA-induced milk fat depression. The lack of an effect on mammary lipogenesis with ROSI could be explained by its insulin-sensitizing properties as suggested previously, that might have increased glucose utilization in peripheral tissues and reducing glucose availability in mammary gland for triglyceride synthesis.

INTRODUCTION

The mammary gland is the most active lipid-synthesizing organ during lactation in mice (Rudolph et al., 2007) as it secretes ~ 30 g of milk lipids over the course of the 20-d lactation period which is equivalent to the dam's entire body weight (Schwertfeger et al., 2003). Milk lipid synthesis involves formation of fatty acids (FA) either *denovo* in the mammary gland or absorption of preformed FA from blood originating in the diet or mobilized from adipose tissue (Smith, 1980). Short-and medium-chain FA (SMCFA) including C8, C10, C12, C14 and half of C16 are synthesized *denovo*, while the rest of the long-chain FA (LCFA; > 16:0) are absorbed preformed from the blood triglycerides (TG). Several mammary lipogenic enzymes are involved in formation of milk fat. Mammary *acetyl-CoA carboxylase (ACC)* and *fatty acid synthase (FASN)* enzymes are involved in the pathway of *denovo* FA synthesis, while *lipoprotein lipase (LPL)* is involved in uptake of FA from triglyTG. Absorbed and *denovo*-synthesized FA (DNFA) are further esterified to glycerol sequentially via *glycerol-3-phosphate acyl transferase (GPAT), acylglycerol-3-phosphate acyl transferase (AGPAT),* and *diacylglycerol acyl transferase (DGAT).* Saturated LCFA (> C14) could be desaturated by the *stearoyl-CoA desaturase (SCD)* before being incorporated into TG (Bernard et al., 2008).

Milk fat synthesis is highly responsive to nutritional manipulation in ruminants (Sutton, 1989). Certain dietary alterations like a high-fat diet or the specific conjugated linoleic acid (CLA) isomer, *trans*-10, *cis*-12 CLA, reduce FA synthesis, causing low milk fat syndrome commonly termed as milk fat depression (MFD) in dairy cows. The CLA-induced MFD is characterized by marked reduction of mRNA abundance of enzymes involved in mammary lipogenesis (Piperova et al., 2000; Peterson et al., 2003) including

major transcription factors such as *sterol regulatory element binding protein (SREBP-1)* (Harvatine and Bauman, 2006) in both dairy cows (Baumgard et al., 2002) and lactating mice (Lin et al., 2004). The CLA-induced MFD alters the milk FA composition by reducing the proportions of SMCFA to a greater extent than LCFA (Loor and Herbein, 1998), suggesting decreased *denovo* FA synthesis. *Trans*-10, *cis*-12 CLA has also been shown to induce insulin resistance associated with macrophage infiltration and adipose tissue lipolysis (Poirier et al., 2005). *Trans*-10, *cis*-12 CLA-induced insulin resistance causes hyperinsulinemia, further triggering hepatic lipid accumulation, leading to hepatic steatosis (Clement et al., 2002; Degrace et al., 2003; Rasooly et al., 2007; Cooper et al., 2008). The intensity of lipid accumulation depends upon the level and duration of feeding, the extent of adipose tissue lipolysis, and the physiological status of the animal (Clement et al., 2002; Vyas et al., 2012).

Peroxisome proliferator activated receptor- γ (PPAR-γ) is an important member of the nuclear receptor super family of transcription factors that can be activated by lipophilic ligands. It regulates adipocyte differentiation and has been implicated as a key protein for thermogenesis and adipose tissue lipid metabolism (Jain et al., 1998). Rosiglitazone (ROSI), a *PPAR-γ* agonist, is commonly used as an insulin sensitizing agent for the treatment of Type-2 diabetes mellitus (Moller, 2001). Liu et al. (2007) observed antagonistic effects of ROSI on *trans*-10, *cis*-12 CLA-induced lipodystrophic disorders and hepatic lipid accumulation.

While the role of *PPAR-γ* has been extensively studied on adipose and hepatic lipid metabolism, its role in the mammary gland is still uncertain (Wan et al., 2007).

Recently, a pivotal role of *PPAR-γ* was observed in maintaining milk quality and protecting newborns by reducing the production of inflammatory lipids in lactating mammary gland (Wan et al., 2007). Bionaz and Loor (2008) suggested that *PPAR- γ* could be the main transcription factor controlling milk fat synthesis by serving as a regulator for *SREBP* activity. Kadegowda et al. (2009) also showed marked upregulation of mammary lipogenic gene expression with ROSI, cultured in bovine mammary epithelial cells.

Because ROSI antagonizes *trans*-10, *cis*-12 CLA-induced adipose tissue lipodystrophy and hepatic steatosis (Liu et al., 2007) and has been shown to upregulate mammary lipogenesis in cell culture, we hypothesized that providing ROSI to lactating females will also antagonize *trans*-10, *cis*-12 CLA-induced milk fat depression by increasing expression of mammary lipogenic enzymes. The main objective of the present study was to study the combination of ROSI and CLA on mammary and hepatic lipogenesis in lactating mice.

MATERIALS AND METHODS

Animals, Diets, and Treatments

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland. Female C57Bl/6J mice (Charles River, Wilmington, MA) were bred to obtain pregnant dams. Day1 of the lactation was the day on which pups were born. Litters were standardized to six pups for all treatments to maintain uniform milk synthesis among dams.

Dams and their pups were housed in shoebox cages and provided with ad-libitum food and water. Pregnant mice were fed a commercial rodent diet (5001 Rodent Lab Diet ®, Purina, Richmond, IN) consisting of 23% CP, 4.5% fat, and 6.0% fiber until 2 d prepartum. From d-2 prepartum until d-6 postpartum, dams were fed a control diet. Twenty-four lactating mice were randomly assigned to one of four treatments (n = 6 per treatment) applied from d-6 to d-10 postpartum. Treatments included: 1) Control diet; 2) Control plus 1.5 % dietary CLA (CLA); 3) Control plus intra-peritoneal (IP) ROSI injections (10 mg/kg BW) (ROSI); and 4) CLA plus ROSI (ROSI-CLA). Mice on the Control and CLA diets received IP injections of phosphate buffered saline (PBS). Daily food intake was recorded during the experimental period. On d-6 and d-10 postpartum, milk samples were collected by suction and milk fat percentages measured as previously described (Teter et al., 1990). Milk samples were stored at -20°C for FA analyses. Body weights of dams and pups were recorded before milking. On d-10 postpartum, the animals were sacrificed using carbon dioxide and individual liver and mammary tissues were collected from dams and livers were collected and pooled from pups within litters from each treatment. Livers were fast-frozen in liquid N_2, and stored at -80°C until RNA, protein, and lipid extraction.

Lipid Extraction and FA Analysis

The FA composition was analyzed from milk samples collected on d-6 and d-10 postpartum and dam and pup liver samples collected on d-10 postpartum. The FA methyl esters (FAME) were prepared by mild transesterification with 1.4 mol/L of H_2SO_4 in methanol (Christie, 1982). Separations were achieved using an Agilent 6890N gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with a flame

ionization detector. Hydrogen was used as carrier gas at 1.6 mL/min with the linear velocity of 26.1 cm/s. Air flow was maintained at 400 mL/min. Nitrogen was used as make up gas with flow rate of 28.4 mL/min. The oven was maintained at 170^{o}C for 50 min followed by the ramp of 6^{o}C per min to 185^{o}C for 50 min with a total run time of 102.5 min. The injection port was maintained at 250^{o}C, and the detector at 250^{o}C. The split ratio was set to 1:100 and the typical injection volume was 1 uL. Individual FA were identified using GLC-60 and GLC-463 standard mixture (Nu-Chek Prep Inc., Elysian, MN).

RNA Isolation and Quantitative Real-Time Reverse-Transcription PCR

Frozen biopsy tissues were weighed (~30 mg) and immediately subjected to RNA extraction using Qiagen RNeasy mini kit with on-column DNAse digestion (Qiagen, Valencia, CA). The RNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.9. The RNA integrity was assessed by electrophoretic analysis of 28S and 18S rRNA subunits using agarose gel electrophoresis.

A portion of the extracted RNA was diluted to 1μg/μL using DNase-RNase free water prior to reverse transcription. The cDNA was synthesized from 1μg RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA) as per the manufacturer's instructions. A negative control to check for genomic DNA contamination was prepared by pooling RNA from each sample, and using 1 μg in a reaction without reverse transcriptase. All first-strand cDNA reactions were diluted 5-fold prior to use in PCR.

The primer sequences utilized in these experiments are detailed in Table 5.3. The samples were run in duplicates and mRNA levels were quantified using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and the 2X Quantitect SYBR Green PCR Master Mix (Bio-Rad). Cycles were performed as follows: denaturation at 95^{o}C for 3 min to activate the polymerase, followed by 40 cycles of 95^{o}C for 15 s, 60^{o}C for 30 s, and 72^{o}C for 30 s. The presence of a single PCR product and the absence of primer-dimers were verified by the melt curve analysis using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were normalized to the housekeeping gene *β-actin (ACTB)* and the specificity of PCR product was verified by separating on 1% agarose gel. The stability of *ACTB* expression was validated by calculating standard deviation (SD), coefficient of variation (CV) and maximum fold change (MFC) (de Jonge et al., 2007). The expression of *ACTB* in liver and mammary tissues had SD of 0.79 and 0.52, CV of 3.96 and 2.71 and MFC of 1.19 and 1.10 respectively. The data were transformed using the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), where Ct represents the fractional cycle number when the amount of amplified product reaches a threshold for fluorescence. The normalized data were transformed to obtain a perfect mean of 1.0 for controls, leaving the proportional difference between the biological replicates. The same proportional change was calculated in all the treatments to obtain a fold change relative to controls.

Protein Isolation and Western Blotting

Protein isolation and western blotting procedures were adapted from (Rudolph et al., 2010). Briefly, mammary lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2.0 mM EDTA, 50 mM NaF, 5.0 mM sodium vanadate, 1% Triton X-100, 1% deoxycholate, and

0.1% SDS] to which 0.57 mM phenylmethylsulfonyl fluoride (PMSF), 20 µL/mL EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1.0 mM DTT were added to extract proteins from the mammary samples. Samples were homogenized using a Brinkman homogenizer, and lysate was centrifuged at 13,000 *g* for 20 min at 4°C. Protein concentrations in the supernatant were determined using bicinchoninic acid (Pierce, Rockford, IL). Proteins were resolved using 8% SDS-polyacrylamide gels (Laemmli, 1970). Resolved proteins were transferred to nitrocellulose membrane (Biorad laboratories, Hercules, CA). Antibodies directed against acetyl-CoA carboxylase-1 (Polyclonal antibodies raised in rabbit; Catalogue # 3662S) and fatty acid synthase (Polyclonal antibodies raised in rabbit; Catalogue # 3180S) and secondary antibodies (Anti-rabbit IgG, HRP linked antibody; Catalogue # 7074S) were obtained from Cell Signaling Technology, Inc. (www.cellsignal.com, Danvers, MA).

Statistical Analyses

Data were analyzed using the GLM procedure in the Statistical Analysis Software (Version 9.2, SAS Institute, Cary, NC). Data from day 6 postpartum were used as a covariate for the analyses of milk fat percentage, milk FA composition, and dam and pup body weight. The statistical model included fixed effect of d 6 values (where appropriate) in the analysis of covariance. A probability of ($P < 0.05$) was considered statistically significant.

RESULTS

Ingredient and FA composition (g/100 g FAME) of the control and CLA diets are presented in Tables 5.1 and 5.2 respectively. The diet was modified from AIN-93 specifications as mentioned earlier (Teter et al., 1990). The major difference in the FA composition of Control and CLA diets was the presence of CLA isomers (mainly *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers). Previous studies have shown that *cis*-9, *trans*-11 CLA isomer had no significant effect on milk fat synthesis and liver and carcass FA composition in lactating mice (Loor et al., 2003); hence the effects of CLA supplementation on mammary and hepatic lipid metabolism were attributed to the presence of *trans*-10, *cis*-12 CLA isomer.

Food Intake, Body and Organ Weights, and Pup Growth Rate

Dam daily food intake, body and liver weights and pup growth rates are presented in Table 5.4. No effects were observed on the average dam body weight at d-10 postpartum. However, CLA reduced ($P < 0.05$) feed intake by 17%. Liver weights were increased ($P < 0.05$) with CLA and the response was more pronounced with control treatment (32%) as compared to CLA-ROSI combination (6%). Similar responses were observed when liver weight was presented as % of body weight (BW). However, ROSI reduced liver weight (as % of BW) ($P < 0.05$) by 10% and 13% when given with control and CLA treatments respectively. The ROSI x CLA interaction tended to rescue CLA-induced increase in liver weight ($P < 0.10$). Liver fat content (measured as FAME per g tissue) was increased ($P < 0.01$) with CLA while no effects were observed with ROSI. However, ROSI tended to reduce ($P < 0.10$) total liver lipids (measured as total FAME).

D-10 pup body weight and growth rate were reduced in both CLA ($P < 0.001$) and ROSI ($P < 0.05$) treatments.

Milk Fat Content and FA Composition

Milk fat content was reduced by 42% with CLA ($P < 0.001$) but not affected by ROSI (Table 5.4). Dietary CLA increased the proportions of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA ($P < 0.001$ and $P < 0.01$ respectively) in total milk FA (Table 5.5). CLA-induced depression in milk fat concentration was accompanied by reduction in total *denovo* synthesized FA (DNFA) content ($P < 0.001$). No effects were observed on short-chain FA (SCFA, 8:0 and 10:0) while CLA reduced medium-chain FA (MCFA, C12 and C14) content ($P < 0.05$; $P < 0.001$, respectively). ROSI reduced ($P < 0.05$) C14 while no effects were observed on other SCFA and MCFA. The CLA-induced decrease in MCFA led to proportional increase in C18:0 ($P < 0.01$) and C18:1*c9* ($P < 0.01$). There was an ROSI x CLA interaction where ROSI increased ($P < 0.05$) 18:1c9 with the CLA treatment but not with the control. The increase in C18 FA with CLA was accompanied with increased total monounsaturated FA (MUFA; $P < 0.05$) and polyunsaturated FA (PUFA; $P < 0.05$) followed by concomitant reduction in saturated FA (SFA) content ($P < 0.05$). The ROSI x CLA interaction was observed for MUFA ($P < 0.05$) and DNFA ($P < 0.10$) where the ROSI-CLA treatment resulted in increased MUFA and decreased DNFA with other treatments.

Hepatic FA Composition

The hepatic FA profile (g/100 g of FAME) in dams is shown in Table 5.6. Conjugated linoleic acid increased the proportions of its constituent isomers, *cis*-9, *trans*-

11 and *trans*-10, *cis*-12 CLA. The CLA diet increased ($P < 0.01$) the proportion of C16:0 and C16:1 while no effects were observed on MCFA (C12 and C14). Similarly, C16:0 content was increased ($P < 0.01$) and C16:1 content tended to increase ($P < 0.10$) with ROSI treatment. *Trans*-10, *cis*-12 CLA-induced increase in C16:0 led to proportionate reduction in the content of C18:0. No CLA effects were observed on C18:1 FA. Rosiglitazone tended to increase ($P < 0.10$) C18:1 content while no effects were observed on C18:0. Polyunsaturated FA content including C20:3, C20:4 and C22:6 were reduced with CLA.

The hepatic FA profiles for pups are presented in Table 5.7. ROSI had no effect on pup hepatic FA composition. However, CLA effects were pronounced on MCFA. Both C12 and C14 were reduced with CLA ($P < 0.01$; $P < 0.05$ respectively). While CLA had no effect on C16:0, the content of C16:1 was reduced ($P < 0.05$). *Trans*-10, *cis*-12 CLA isomer was transferred from dam milk to pup liver as its content was increased ($P < 0.05$) with CLA. The concentration of *cis*-9, *trans*-11 CLA was similar across all treatments.

Mammary Lipogenic Gene Expression

Mammary lipogenic gene expression data in is presented in Figures 5.1, 5.2, and 5.3. The mRNA abundance of mammary lipogenic genes coincide with milk fat responses observed with CLA as the enzymes involved in *denovo* FA synthesis were reduced. While CLA effects were significant for *FASN* ($P = 0.002$), the *ACC* mRNA abundance only tended ($P = 0.09$) to be reduced with CLA. The mRNA expression of enzymes involved in FA uptake (*LPL*), desaturation (*SCD-1*), and TG synthesis (*DGAT)*

were reduced with CLA ($P = 0.06$; $P < 0.001$; $P < 0.01$ respectively). The mRNA expression of the transcription factors *SREBP-1c* ($P < 0.001$) and *PPAR-γ* ($P = 0.002$) and transcription co-activators *INSIG-1* ($P < 0.001$), *SCAP* ($P < 0.001$) and *RXR* ($P < 0.001$) were also reduced with CLA. No ROSI effects were observed on mRNA expression of mammary lipogenic enzymes, *SREBP-1c* and *SREBP-1c* co-activators *SCAP* and *INSIG-1*. There was a trend for a ROSI by CLA interaction where ROSI tended to increase the expression of *PPAR-γ* ($P < 0.10$) and *RXR* ($P < 0.10$) compared with CLA alone.

Hepatic Lipogenic Gene Expression

Hepatic lipogenic gene expression responses are presented in Figures 5.4, 5.5 and 5.6. Conjugated linoleic acid treatment reduced hepatic *ACC* expression ($P = 0.01$) while no effects were observed on *FASN*. The combination of ROSI along with CLA further tended to reduce *ACC* expression ($P = 0.07$) while the expression of *FASN* was reduced ($P = 0.01$) compared with other treatments. Similarly, there was a ROSI by CLA interaction where CLA alone increased hepatic *LPL* expression but not in presence of ROSI. The expression of *SCD*-1 was reduced by CLA ($P = 0.02$) while ROSI increased the *DGAT* mRNA expression ($P = 0.02$). Among transcription factors, expression of *SREBP-1c* was reduced ($P < 0.05$) with CLA while ROSI x CLA interaction tended to decrease ($P < 0.10$) *PPAR-γ* expression compared to CLA and ROSI alone. Transcription activators *INSIG-1*, *SCAP* and *RXR* were not affected by treatments.

Mammary and hepatic protein expression

The mammary and hepatic protein abundance for ACC and FASN relative to the levels of β-tubulin are presented in Figure 5.7 and 5.8, respectively. The protein abundance of ACC and FASN in mammary gland was ($P < 0.01$) reduced with CLA while no effects were observed with ROSI. Mammary protein abundance for ACC and FASN mirrored gene expression responses shown in Figure 5.1 but not hepatic gene expression for the enzymes shown in Figure 5.4.

DISCUSSION

The role of *trans*-10, *cis*-12 CLA in suppressing mammary lipogenesis has been extensively studied (Bauman et al., 2011). The coordinated downregulation of mammary lipogenic genes suggested an important role of transcription factor, *SREBP-1c* during milk fat synthesis (Harvatine and Bauman, 2006). However, recently the role of *PPAR-γ,* a member of nuclear receptor family of transcription factor, was suggested as a potential regulator of mammary lipogenesis (Bionaz and Loor, 2008). That assumption was supported by recent findings where mammary lipogenesis was upregulated with ROSI, a *PPAR-γ* agonist, in bovine mammary epithelial cells (Kadegowda et al., 2009).

Dietary CLA reduced dam food intake but no effects were observed of reduced food consumption for body weight. The food intake effects of *trans*-10, *cis*-12 CLA were comparable to previous reports with lactating mice (Park et al., 1999; Loor et al., 2003). The effects of *trans*-10, *cis*-12 CLA on dam body weight have been consistent. Body weight was reduced by 17% in some (Loor et al., 2003) while no effect was observed in others (Kadegowda et al., 2010). The responses on body weight vary depending on level

and duration of *trans*-10, *cis*-12 CLA feeding, extent of adipose tissue lipolysis, and physiological stage of animal (Park et al., 1997; Clement et al., 2002; Larsen et al., 2003; Vyas et al., 2012). Dietary *trans*-10, *cis*-12 CLA supplement was fed from d-6 to d-10 in the present study at dose rate of 0.6% of total diet and the response was comparable to the study with a similar dose and feeding duration (Kadegowda et al., 2010). However, dam body weight reduced by 35% when *trans*-10, *cis*-12 CLA was fed from d 4 to d 15 postpartum at dose rate of 0.96% of total diet (Loor et al., 2003). In addition to dose and duration, CLA reduced milk fat secretion, thereby reducing energy needs of the dam such that BW can be maintained in the face of reduced food intake. The growth rate in pups from dams fed CLA diet was reduced indirectly perhaps due to reduced energy intake. However, hepatic FA profile in pups from dams fed CLA diet showed increased *trans*-10, *cis*-12 CLA from suggesting direct effects of CLA on pup growth rate. The results were comparable to previous studies (Loor et al., 2003; Kadegowda et al., 2010). However, some studies in lactating rats showed improved pup growth rate with CLA (Poulos et al., 2001). The inconsistencies in the growth rate response could be attributed to species-specific effects of CLA.

While CLA reduced milk fat content in a manner comparable to previous studies (Loor et al., 2003; Kadegowda et al., 2010) there were no effects of ROSI or ROSI x CLA interaction on milk fat responses. Reduced milk fat content with CLA diet was accompanied by reduced proportions of DNFA including C12 and C14 suggesting inhibition of *denovo* FA synthesis. The mRNA and protein expression of *ACC* and *FASN*, ,critical enzymes catalyzing *denovo* FA synthesis, were also reduced further reflecting the CLA-induced changes observed in milk FA composition. Along with FA synthesis, the

mRNA abundance of genes regulating FA uptake (*LPL)* was also reduced suggesting reduced uptake of preformed FA from blood TG.

The CLA-induced inhibition of FA desaturation was demonstrated by reduced mRNA abundance of *SCD-1*, along with the ratio of 18:1c9/18:0 providing indirect evidence of reduced *SCD-1* activity. These desaturation effects were comparable to those with previous studies in lactating mice (Lin et al., 2004).

Trans-10, *cis*-12 CLA reduced the mRNA expression of *DGAT-1*, an enzyme catalyzing the final and committed step in the process of TG synthesis (Sorensen et al., 2008). However, no CLA effect was observed on *DGAT-1* mRNA expression in MACT cells (Sorensen et al., 2008). In the same study the enzyme activity was reduced. The difference in both studies could be attributed to different models used for studying milk fat synthesis. The animal model used in the present study is more representative of the biological system as compared to cell lines used earlier (Sorensen et al., 2008).

Although the molecular mechanisms regulating milk fat synthesis are not well established, the coordinated down-regulation of mammary lipogenesis suggests the involvement of transcription regulation (Harvatine and Bauman, 2006). The role of *SREBP-1* has been suggested as a global regulator of mammary lipid metabolism (Rudolph et al., 2007) and is synthesized as precursor protein associated with *SCAP* and anchored to the endoplasmic reticulum with *INSIG* protein. Upon activation, the *SREBP-1* precursor undergoes a sequential two step cleavage process and the mature protein is translocated to the nucleus where it binds the target genes on sterol response elements (Sakai et al., 1998, Wang et al., 1994). The mRNA expression of *SREBP-1c* along with

SCAP and *INSIG-1* were reduced with CLA. The results are comparable to previous studies with *trans*-10, *cis*-12 CLA in cows (Harvatine and Bauman, 2006; Kadegowda et al., 2010) suggesting both transcriptional and post-transcriptional modification of *SREBP-1c* synthesis.

Gene expression of *PPAR-γ* was reduced with CLA in mammary gland. As expected, ROSI increased *PPAR-γ* expression and there was as ROSI x CLA interaction where the reduction due to CLA was modulated by ROSI. The role of *PPAR-γ* has been extensively studied in relation to adipose and hepatic lipid metabolism but its effect on mammary lipid metabolism is not well documented. Present study has provided little evidence of its involvement in CLA-induced MFD.

The lack of ROSI effects on mammary lipogenesis might be attributed to its insulin sensitizing properties. Glucose is required for synthesis of free FA in mice mammary gland (Anderson et al., 2007). Previous studies using ^{14}C have shown that 40-70% of fatty acid synthesized is derived from glucose metabolized through pentose phosphate pathway (Abraham and Chaikoff, 1959). Rosiglitazone increases the peripheral utilization of glucose (Ye et al., 2004) thereby reducing the glucose availability as carbon source for DNFA synthesis in mammary gland.

The effects of CLA and ROSI on hepatic lipid metabolism in the present study were determined by measuring dam liver weight and FA composition along with lipogenic gene and protein expression. Dietary CLA increased the liver weight due to increased lipid accumulation. The response is comparable to previous study using *trans*-10, *cis*-12 CLA in lactating mice (Kadegowda et al., 2010). Increased lipid accumulation

can be attributed to various factors including increased FA influx, increased FA synthesis and reduced FA oxidation and TG secretion (Jourdan et al., 2009). However mRNA expression profile suggests a compensatory response to counteract CLA-induced elevated lipid accumulation. The mRNA abundance of *ACC* along with *SCD* and *SREBP-1c* were reduced while no effects were observed on *FASN*, *LPL*, and *DGAT* expression with dietary CLA. Previous studies with liver specific *ACC -1* knockout mice failed to be protected against high fat/high carbohydrate diet-induced obesity and fatty liver (Mao et al., 2006) due to a compensatory increase in the expression of *ACC-2.* Similarly, tissue specific *FASN* knockout did not protect against the development of fatty liver but rather exacerbated it by reducing FA oxidation (Chakravarthy et al., 2005). While the gene expression involved in lipogenesis was reduced in the present study with CLA, the increased hepatic lipid accumulation might have resulted from either increased uptake of FA, reduced FA oxidation or secretion (Vyas et al., 2012). The responses observed in this study contrast with those in previous studies with lactating mice (Lin et al., 2004; Kadegowda et al., 2010) where the hepatic lipogenic gene expression remained unaltered in response to *trans*-10, *cis*-12 CLA. Rosiglitazone rescued *trans*-10, *cis*-12 CLA-induced hepatic steatosis. These results were comparable to earlier study demonstrating antagonistic effects of ROSI on *trans*-10, *cis*-12 CLA-induced adipose tissue lipodystrophy and hepatic steatosis (Liu et al., 2007). The reduced hepatic lipid accumulation with ROSI x CLA interaction correlates well with the lipogenic gene expression. The mRNA expression of *FASN* was reduced while that of *ACC* tended to reduce with the combination of ROSI and CLA. Findings are comparable to previous

study studying combination of ROSI and CLA on hepatic lipid metabolism (Liu et al., 2007).

The effects of CLA and ROSI can further be elucidated with the hepatic FA profile. The hepatic FA composition during non-alcoholic fatty liver disease (NAFLD) is characterized by substantial reductions in long chain polyunsaturated FA (LC-PUFA) concentrations; specifically that of arachidonic acid (20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Belury and KempaSteczko, 1997; Sebedio et al., 2001; Chardigny et al., 2003; Kelley et al., 2004; Kelley et al., 2006; Kadegowda et al., 2010; Martins et al., 2011). The hepatic FA profile in CLA fed mice in the present study was similar to that observed during NAFLD with reduced LC-PUFA concentrations.

It has been shown that normalizing the levels of LC-PUFA can ameliorate hepatic steatosis when supplemented along with CLA. Supplementing arachidonic acid (Oikawa et al., 2009) or its precursor γ-linolenic acid (18:3 n-6) (Nakanishi et al., 2004) decreased induction of hepatic steatosis. Similarly, supplementing 20:5n-3 and 22:6n-3 prevents lipid accumulation when fed with *trans*-10, *cis*-12 CLA (Vemuri et al., 2007, Yanagita et al., 2005). In the present study the concentrations of LC-PUFA including 18:2n-6, 20:3 and 22:6 were increased while the concentrations of 20:4 and 20:5 tended to increase with ROSI suggesting mechanism behind preventing hepatic steatosis.

CONCLUSION

Trans-10, *cis*-12 CLA-induced MFD in lactating mice was also associated with increased lipid accumulation in liver leading to hepatic steatosis. Administration of ROSI had no effect on mammary lipogenesis and failed to rescue CLA-induced MFD. However ROSI prevented CLA-induced hepatic steatosis.

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Table 5.1. Composition of experimental diets fed to lactating mice[1]

Ingredients	Control	CLA
Sucrose	62.95	62.95
Soybean oil	7	5.5
Solka floc[2]	5	5
AIN-93 Mineral mix[2]	3.5	3.5
AIN-93 Vitamin mix	1	1
L-Cystine	0.3	0.3
Choline biTartarate	0.25	0.25
Casein[2]	20	20
Clarinol[3]	------	1.5
Water	Q.S.	Q.S.

[1] Modified from AIN-93 specifications
[2] ICN biomedicals
[3] Lipid Nutrition, Maywood, NJ

Table 5.2. Fatty acid composition (g/100 g FAME) of lipids added to diets of lactating mice

FA	Control[1]	CLA[2]
8:0	0.01	0.01
10:0	0.04	0.04
12:0	0.06	0.06
14:0	0.26	0.24
15:0	0.04	0.03
14:1	0.02	0.01
16:0	10.94	8.06
16:1	0.11	0.10
17:0	0.12	0.10
17:1	0.06	0.05
18:0	3.92	2.88
18:1 *cis*-9	21.39	17.89
18:1 *cis*-11	1.41	1.09
18:2	52.45	41.05
18:3	7.00	5.04
20:1	0.21	0.16
18:2 *trans*-8,*cis*-10	0.02	0.33
18:2 *trans*-9,*cis*-11	0.03	0.30
18:2, *cis*-9,*trans*-11	0.08	9.84
18:2, *trans, trans*	0.09	0.83
18:2, *trans*-10,*cis*-12	ND[3]	10.54
C22:6	0.03	0.03

[1] Soybean oil (7% of diet)
[2] Soybean oil + Clarinol (5.5% and 1.5% of diet respectively)
[3] ND, Not detected <0.005 g/100 g FAME

Table 5.3. Primers used for RT-PCR analysis

Gene	Accession #		Primers (5'-3')[1]	bp[2]	Reference
FASN	NM_00798	F	ACCTCTCCCAGGTGTGTGAC	106	Kadegowda et al., 2010
		R	TGGATGATGTTGATGATGG		
ACACA	NM_13360	F	GAAAATCCACAATGCCAAC	106	Kadegowda et al., 2010
		R	GTCCCAGACGTAAGCCTTCA		
SCD-1	NM_00912	F	TCCAGTGAGGTGGTGTGAAA	124	Kadegowda et al., 2010
		R	TTATCTCTGGGGTGGGTTTG		
LPL	NM_008509	F	AGCCCTTGCTAGGAGAAAGC	119	Kadegowda et al., 2010
		R	GGGATGCCGGTAACAAATT		
SREBP-1c	NM_011480	F	GTGAGCCTGACAAGCAATCA	103	Kadegowda et al., 2010
		R	GGTGCCTACAGAGCAAGAG		
PPAR-G	NM_01114	F	TGCAGCTCAAGCTGAATCA	94	Kadegowda et al., 2010
		R	ACGTGCTCTGTGACGATCTG		
RXR	NM_011305	F	TCCTTGGGAGGGTCTTCTCT	107	Kadegowda et al., 2010
		R	GGGCAGGTAGCAACACAGA		
SCAP	NM_001144	F	TCAGCCAAACATTTGCTCA	106	Kadegowda et al., 2010
		R	CTGCGGTCCCAGATACTGA		
INSIG-1	NM_15352	F	TGAGTCGCTGTCTGCTGTTT	105	Kadegowda et al., 2010
		R	TCACAGATTGCAAGCTCCAC		
ACTB	NM_007393.3	F	AGCCATGTACGTAGCCAT CC	228	This study
		R	CTCTCAGCTGTGGTGGTGAA		

[1] Primer direction (F – forward; R – reverse)
[2] Amplicon size in base pair (bp)

Table 5.4. Effects of different treatments on dam body weight, food intake, milk fat, liver weight and pup growth rate

Item	Treatments				SEM	*P* values		
	Control	CLA	ROSI	ROSI-CLA		ROSI	CLA	ROSI x CLA
Dams[2]								
n	6	6	6	6				
Body weight, g	27.8	27.2	27.3	28.1	0.59	0.576	0.992	0.288
Intake, g/d	7.02	5.77	7.60	6.36	0.496	0.255	0.021	0.993
Milk fat, % [2]	34.4	21.3	34.2	19.0	1.112	0.255	<0.001	0.301
Liver wt., g	2.03	2.67	2.16	2.30	0.122	0.350	0.026	0.092
Liver wt., % of BW	8.31	9.76	7.50	8.53	0.399	0.014	0.006	0.573
Liver FAME[3], mg/g tissue	87.5	160.0	67.0	119.8	23.24	0.215	0.019	0.680
Total liver FAME, g	0.21	0.43	0.14	0.25	0.632	0.074	0.019	0.375
Pups (n=6 / litter)								
Day 10 pup weight, g	5.29	4.74	5.04	4.28	0.142	0.012	0.001	0.463
Pup weight gain, g/d	0.50	0.22	0.39	0.20	0.034	0.073	<0.001	0.196

[1] Values are least squares means ± SEM.
[2] Volume percent (ml/100ml milk)
[3] Fatty acid methyl esters

Table 5.5. Effects of different treatments on milk fatty acid composition in lactating mice

Fatty acid	Treatments				SEM	*P*-Value		
	CON	CLA	ROSI	ROSI-CLA		ROSI	CLA	ROSI x CLA
	g/100 g of FAME[1]							
8:0	0.11	0.21	0.16	0.13	0.075	0.741	0.448	0.341
10:0	2.81	2.71	2.47	2.48	0.947	0.772	0.964	0.951
12:0	7.21	4.98	5.87	3.87	0.884	0.202	0.044	0.902
12:1	0.08	0.02	0.10	0.02	0.006	0.028	<0.001	0.121
14:0	11.4	5.6	10.0	4.3	0.560	0.039	<0.001	0.936
14:1(*c9)*	0.24	0.04	0.20	0.07	0.046	0.396	0.019	0.563
15:0	0.02	0.03	0.02	0.06	0.019	0.220	0.252	0.301
16:0	30.1	30.8	41.2	26.3	5.72	0.580	0.252	0.210
16:1 (*c9)*	2.77	1.79	2.46	2.95	0.310	0.204	0.449	0.043
17:0	0.40	0.69	0.36	0.80	0.032	0.317	<0.001	0.048
18:0	1.97	4.69	2.26	3.92	0.610	0.702	0.007	0.406
18:1(*c9)*	19.8	21.6	15.8	26.0	1.39	0.877	0.002	0.017
18:1 (*c11)*	2.03	1.91	1.63	2.47	0.160	0.637	0.047	0.015
18:2 (*c9c12)*	13.5	14.7	11.4	14.9	1.52	0.512	0.161	0.477
18:2 (*c9t11)*	1.09	1.96	0.98	2.70	0.352	0.390	0.006	0.253
18:2 (*c11t13)*	ND[2]	0.02	ND	0.04	0.01	0.66	<0.001	0.66
18:2 *(t8c10)*	ND	0.04	ND	0.07	0.027	0.564	0.031	0.564
18:2 *(t9c11)*	ND	0.04	ND	0.06	0.020	0.615	0.001	0.483
18:2 *(t10c12)*	ND	1.68	ND	1.48	0.231	0.64	<0.001	0.65
18:2 *(t,t)*	ND	0.31	ND	0.26	0.052	0.54	0.002	0.48
18:3 *(c6,c9,c12)*	0.11	0.29	0.13	0.31	0.026	0.590	<0.001	0.977

18:3 *(c9,c12,c15)*	1.14	1.08	0.90	1.00	0.161	0.341	0.935	0.629
20:1 *(c-9)*	0.76	0.59	0.61	0.55	0.051	0.100	0.069	0.320
20:3 *(c8,c11,c14)*	0.68	0.25	0.68	0.38	0.091	0.470	0.003	0.522
20:4	0.49	0.95	0.51	1.01	0.152	0.798	0.020	0.881
20:5	0.13	0.15	0.14	0.23	0.053	0.412	0.252	0.440
22:6	0.25	0.42	0.28	0.39	0.071	0.963	0.074	0.684
Other	2.94	2.48	2.00	3.25	0.11	0.99	0.71	0.51
DNFA[3]	36.8	28.9	39.2	24.0	1.76	0.492	<0.001	0.07
MUFA[4]	23.9	24.0	19.4	29.7	1.81	0.763	0.021	0.021
PUFA[5]	17.5	21.9	15.0	22.8	2.12	0.711	0.021	0.456
SFA[6]	53.9	49.6	62.2	41.8	4.59	0.965	0.027	0.118
<16:0	22.1	13.5	18.8	10.9	2.43	0.262	0.010	0.905
16:0	30.06	30.80	41.19	26.34	5.72	0.58	0.25	0.21
>16:0	42.7	51.5	36.0	56.8	3.05	0.823	0.001	0.082

[1] Fatty acid methyl esters

[2] ND, Not detected < 0.005 g/100 g FAME

[3] *Denovo* synthesized fatty acids

[4] Monounsaturated fatty acids

[5] Polyunsaturated fatty acids

[6] Saturated fatty acids

Table 5.6. Effects of different treatments on hepatic fatty acid composition in lactating mice

Fatty acid	Treatments				SEM	*P*-Value		
	CON	CLA	ROSI	ROSI-CLA		ROSI	CLA	ROSI x CLA
	g/100 g of FAME[1]							
12:0	0.02	0.03	0.03	0.02	0.007	0.210	0.282	0.111
14:0	0.82	0.82	0.85	1.19	0.330	0.322	0.294	0.608
16:0	20.0	26.4	17.1	21.7	1.98	0.021	0.008	0.598
16:1 (*c7)*	1.11	1.25	0.80	0.97	0.187	0.074	0.370	0.930
16:1 (*c9)*	1.16	2.25	1.12	1.84	0.230	0.083	0.001	0.344
18:0	14.4	8.9	15.8	10.8	2.40	0.236	0.030	0.904
18:1(*c9)*	35.5	42.3	22.0	33.9	6.87	0.058	0.156	0.670
18:1 (*c11)*	3.68	4.13	3.47	3.89	0.544	0.523	0.360	0.977
18:2 (*c9c12)*	11.0	7.40	14.2	7.90	0.933	0.007	<0.001	0.131
18:2 (*c9t11)*	0.66	0.79	0.45	0.85	0.19	0.444	0.162	0.410
18:2 *(t10c12)*	ND[2]	0.22	ND	0.24	0.01	0.722	<0.001	0.133
18:3 *(c9,c12,c15)*	0.16	0.17	0.33	0.15	0.049	0.041	0.123	0.051
20:1	1.24	0.83	0.66	0.73	0.240	0.127	0.310	0.260
20:3 *(c8,c11,c14)*	0.83	0.29	1.46	0.54	0.047	<0.001	<0.001	0.005
20:4	5.42	1.63	7.62	3.12	2.17	0.09	0.003	0.152
20:5	0.02	0.02	0.04	0.05	0.010	0.070	0.012	0.423
22:0	0.14	0.06	0.08	0.08	0.050	0.731	0.241	0.400
22:6	2.15	0.59	6.62	1.01	1.306	0.021	0.017	0.103

[1] Fatty acid methyl esters

[2] ND- Not detected

Table 5.7. Effects of different treatments on pup liver fatty acid composition

Fatty acid	Treatments				SEM	*P*-Value		
	CON	CLA	ROSI	ROSI-CLA		ROSI	CLA	ROSI*CLA
	g/100 g of FAME[1]							
12:0	0.69	0.15	0.62	0.14	0.048	0.503	0.003	0.680
14:0	2.20	0.74	2.43	0.56	0.300	0.454	0.013	0.580
16:0	23.6	23.2	24.1	21.0	2.20	0.770	0.469	0.630
16:1 (*c9)*	0.75	0.55	1.00	0.38	0.082	0.440	0.013	0.103
18:0	11.90	17.68	10.02	15.80	2.41	0.220	0.053	0.998
18:1(*c9)*	10.41	11.42	13.81	9.20	1.99	0.858	0.360	0.300
18:1 (*c11)*	1.79	1.90	1.82	1.78	0.171	0.775	0.953	0.730
18:2 (*c9c12)*	16.7	16.0	20.1	13.6	1.26	0.675	0.060	0.129
18:2 (*c9t11)*	0.77	1.12	0.76	0.61	0.302	0.272	0.850	0.382
18:2 *(t10c12)*	ND[2]	0.35	ND	0.29	0.061	0.230	0.014	0.647
18:3 *(c9,c12,c15)*	0.47	0.31	0.55	0.20	0.04	0.687	0.007	0.111
20:4	11.98	13.12	9.90	12.62	1.352	0.386	0.254	0.636
20:5	0.28	0.26	0.35	0.24	0.062	0.693	0.352	0.616
22:0	0.04	0.04	0.04	0.02	0.008	0.512	0.179	0.343
22:4	0.62	0.45	0.34	0.36	0.280	0.507	0.820	0.711
22:5	0.86	0.51	0.79	0.48	0.134	0.951	0.052	0.882
22:6	10.18	8.00	8.65	7.06	2.61	0.692	0.434	0.896

[1] Fatty acid methyl esters

[2] ND- Not detected

Figure 5.1. Relative mRNA abundance of mammary lipogenic enzymes *acetyl-CoA carboxylase (ACC), fatty acid synthase, lipoprotein lipase (LPL)* and *stearoyl-CoA desaturase (SCD)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

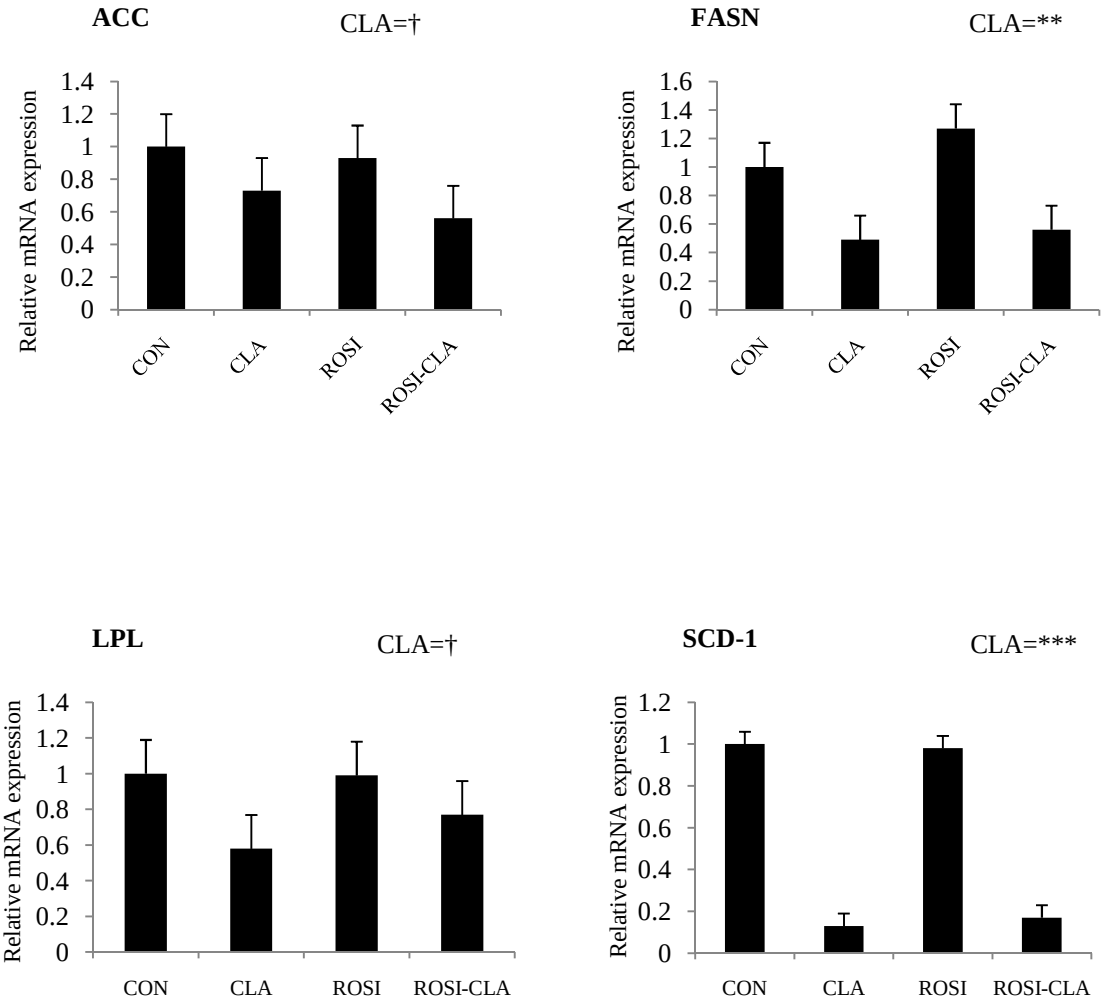

Figure 5.2. Relative mRNA abundance of mammary *diacylglycerol acyl transferase (DGAT), sterol regulatory element binding protein-1 (SREBP-1), peroxisome proliferator activated receptor (PPAR-γ)* and *insulin induced gene-1 (INSIG-1)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

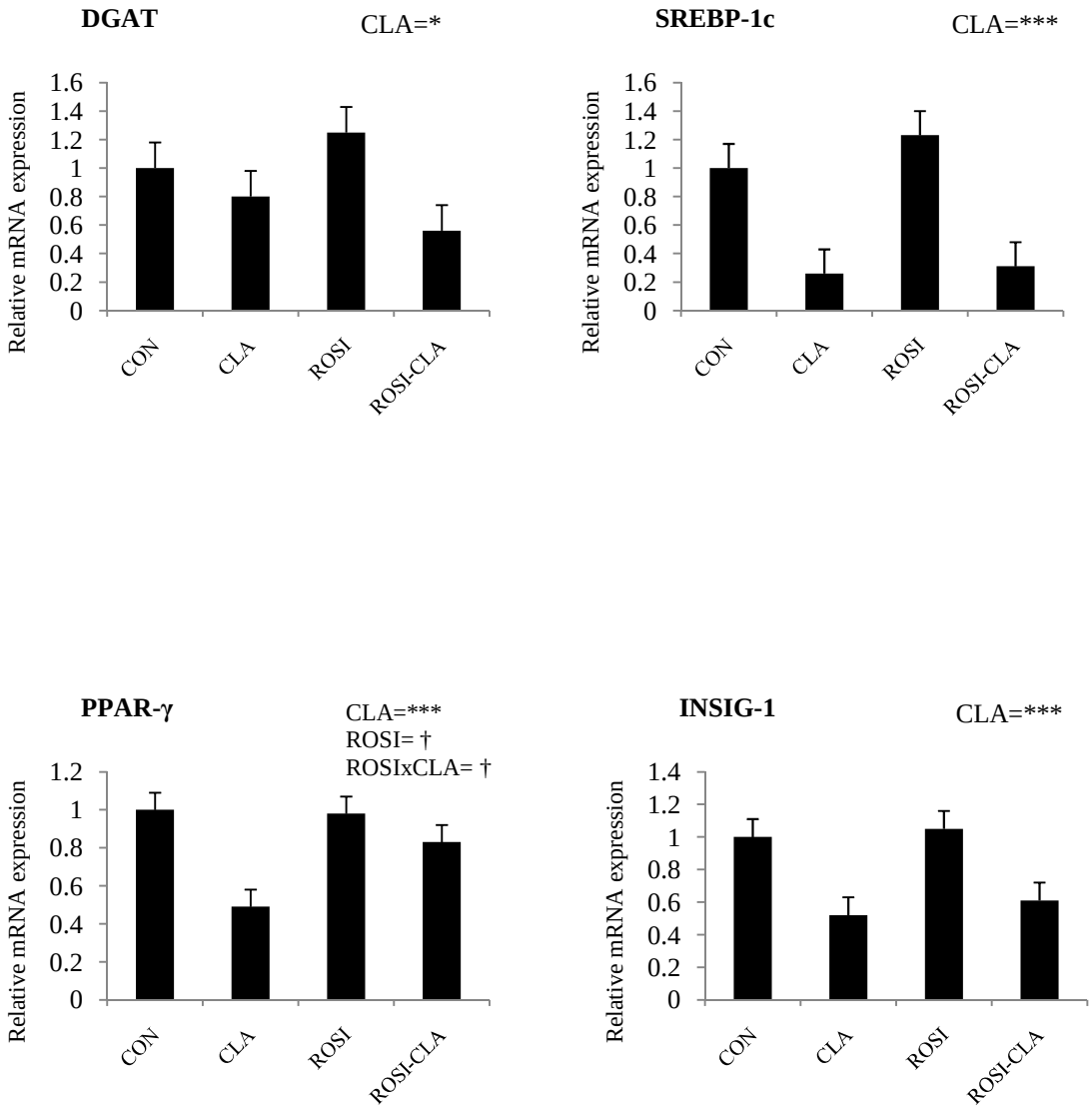

Figure 5.3. Relative mRNA abundance of mammary *SREBP cleavage activating protein (SCAP)* and *retinoid X receptor (RXR)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

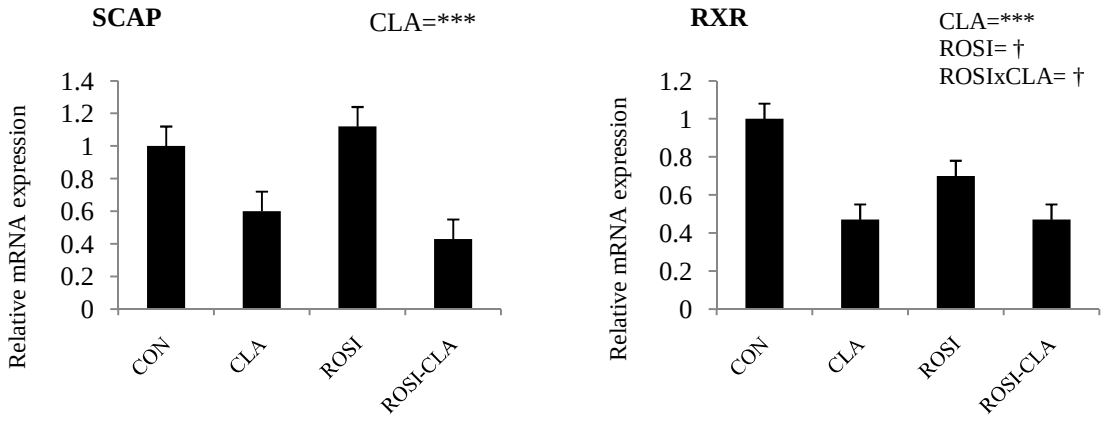

Figure 5.4. Relative mRNA abundance of hepatic lipogenic enzymes *acetyl-CoA carboxylase (ACC), fatty acid synthase, lipoprotein lipase (LPL)* and *stearoyl-CoA desaturase (SCD)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

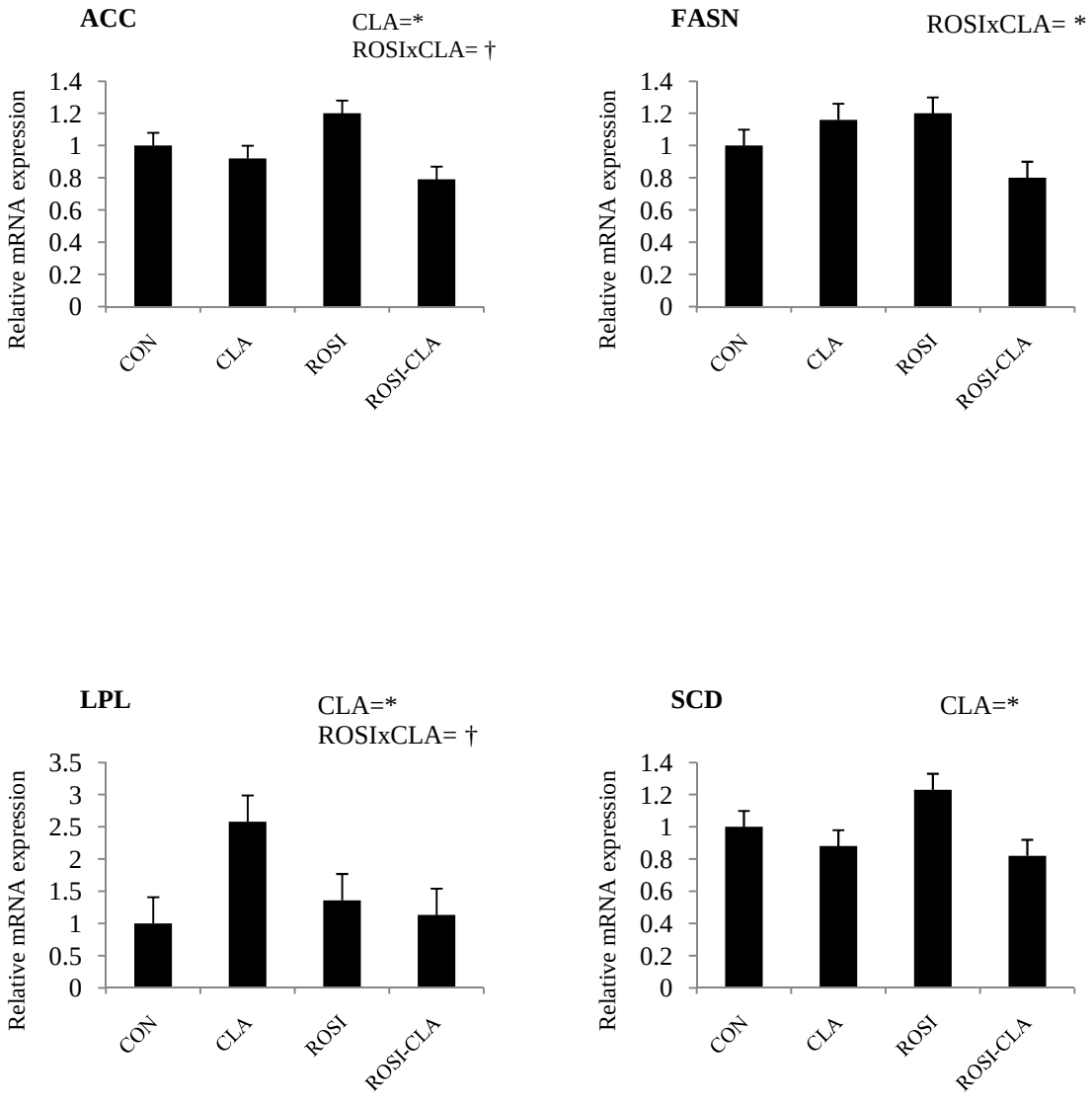

Figure 5.5. Relative mRNA abundance of hepatic *diacylglycerol acyl transferase (DGAT), sterol regulatory element binding protein-1 (SREBP-1), peroxisosome proliferator activated receptor (PPAR-γ)* and *SREBP cleavage activating protein (SCAP)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

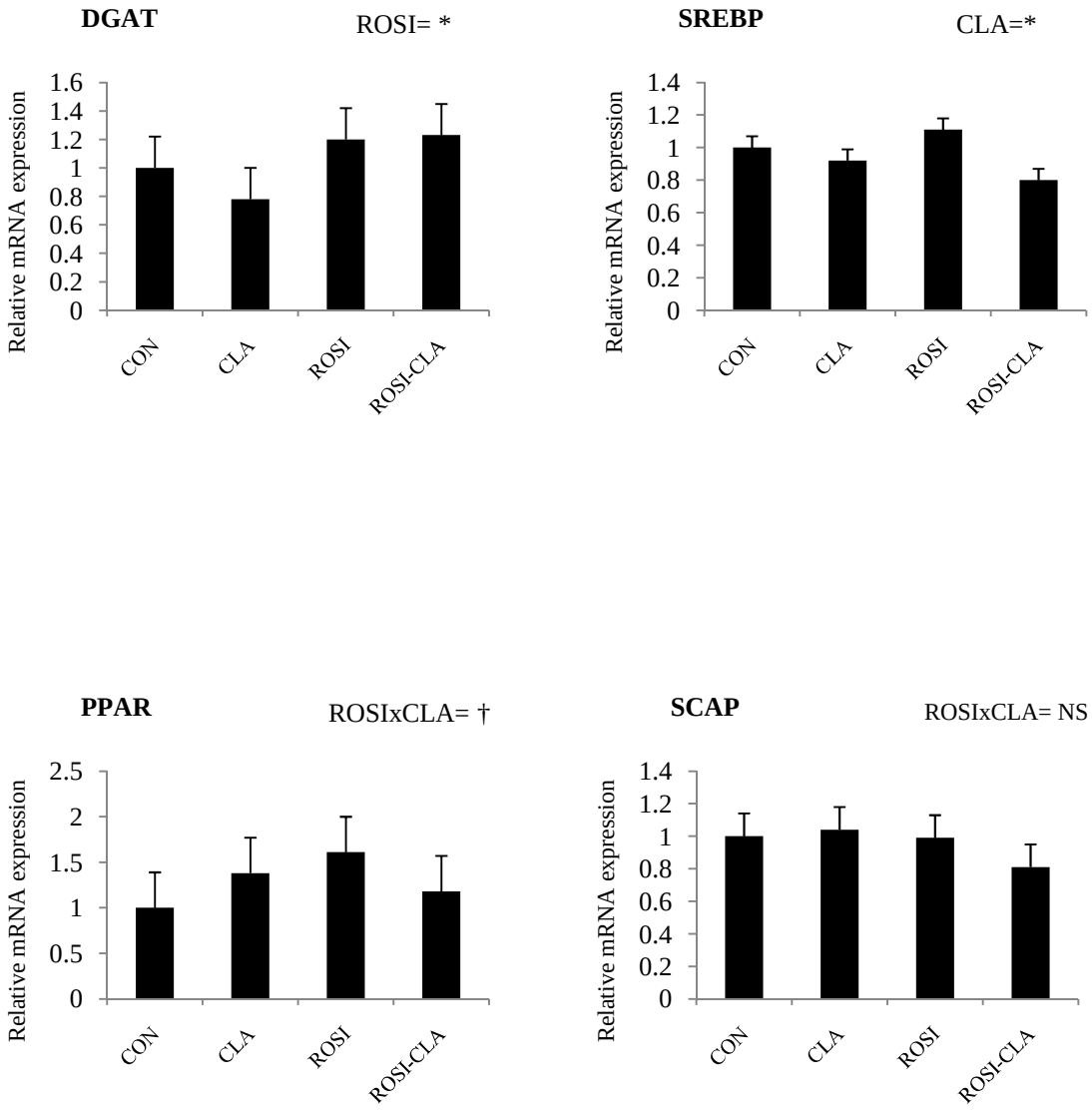

Figure 5.6. Relative mRNA abundance of hepatic *insulin induced gene-1 (INSIG-1)* and *retinoid X receptor (RXR)* in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

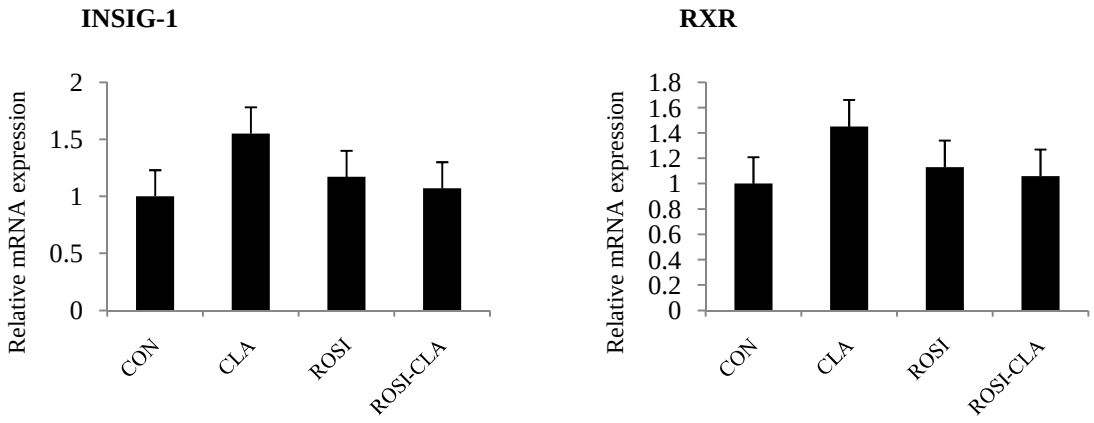

Figure 5.7. Relative protein abundance of mammary acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)

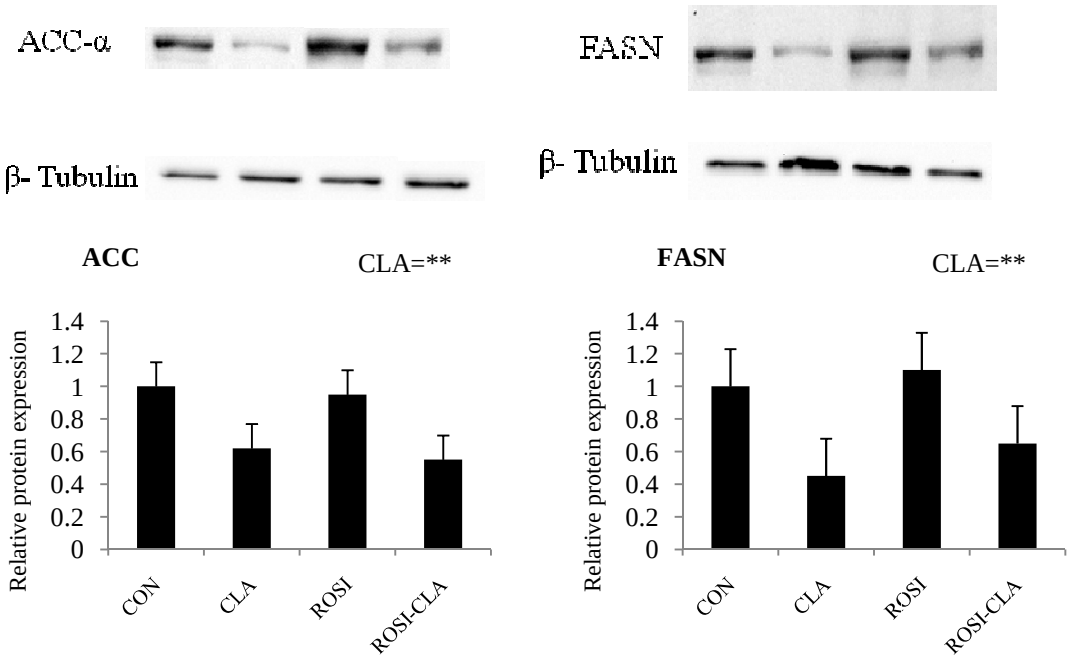

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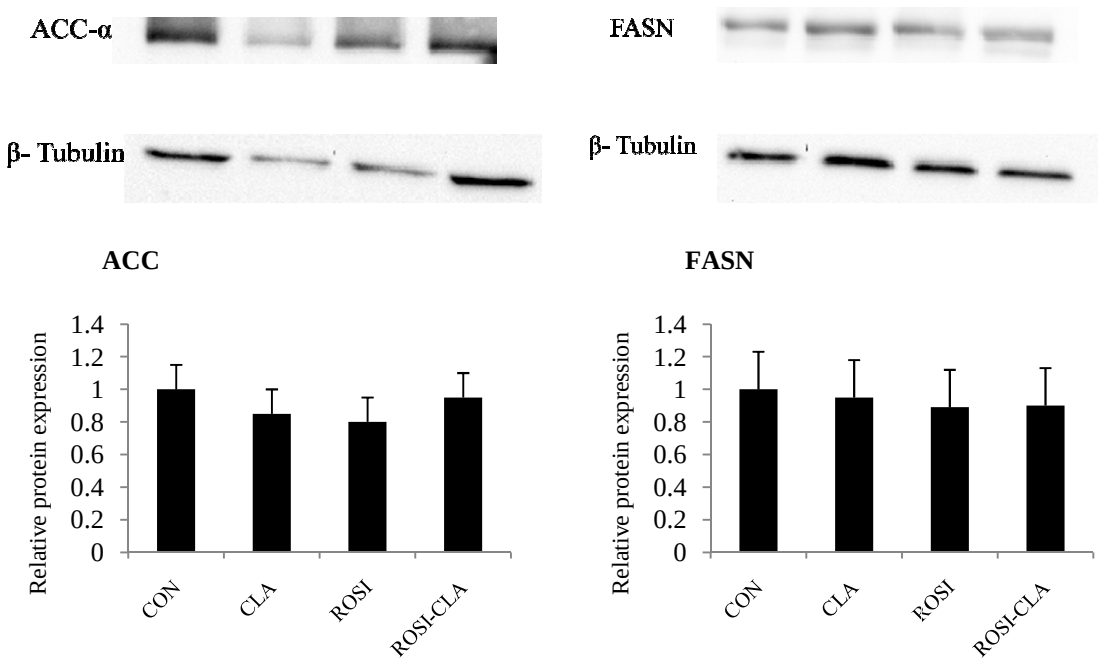
Figure 5.8. Relative protein abundance of hepatic acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) in response to different treatments. Data are expressed as relative to control fed mice (n=6 mice per treatment group; † $P < 0.1$;* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$)



Chapter 6: SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The overall objective of the dissertation was to study the potential limitation of *denovo* synthesized fatty acids (DNFA) during milk fat synthesis. In the first study, the availability of SMCFA, added in proportion as synthesized *denovo* in the mammary gland, was increased via dietary supplementation in lactating dairy cows. In a subsequent study, butterfat (BF), used as a source of SMCFA, was abomasally infused during conjugated linoleic acid (CLA)-induced milk fat depression (MFD) in lactating dairy cows. Finally, Rosiglitazone (ROSI), a *peroxisome proliferator activated receptor-γ (PPAR- γ)* agonist, was used in lactating mice, in an effort to upregulate *denovo* fatty acid (FA) synthesis during CLA-induced MFD.

The results from the studies demonstrated small and non-significant changes in milk fat output in response to SMCFA. Dietary supplementation of SMCFA had no effect on milk fat yield (Figure 6.1). The lack of milk fat response could be attributed to reduced milk yield at higher levels of SMCFA supplementation. The inefficient transfer efficiency of short chain FA (SCFA) including C8 and C10 possibly due to their preferential utilization as energy substrates might also have contributed to lack of milk fat responses.

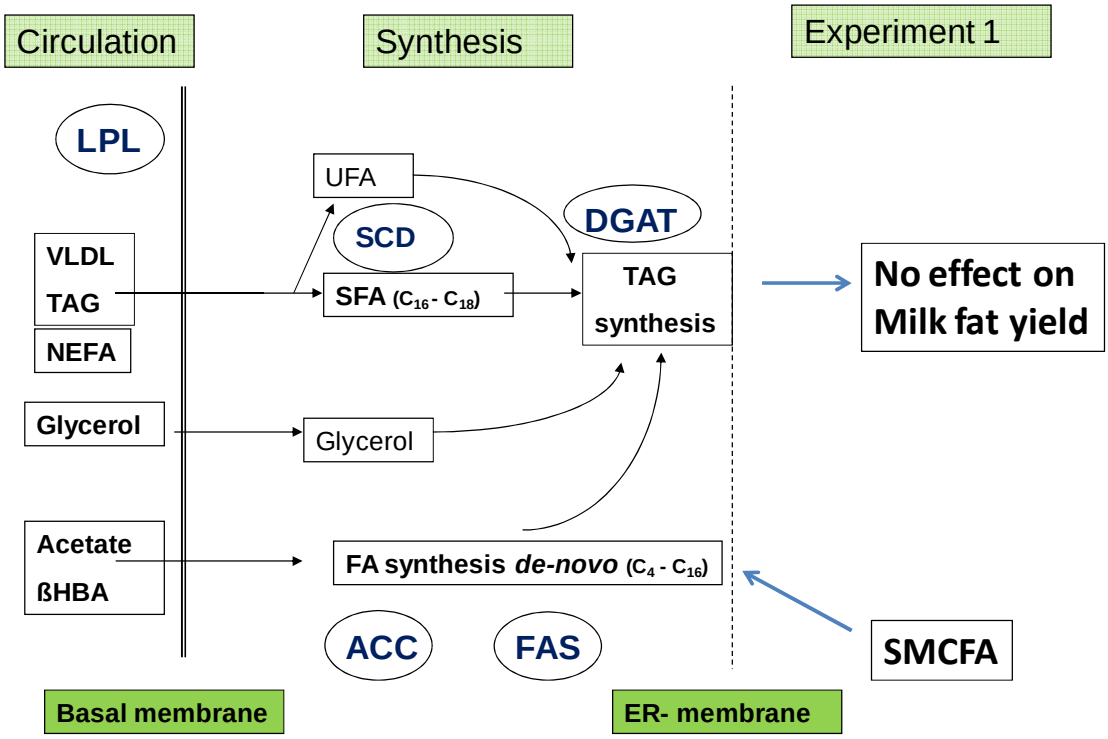


Figure 6.1. The effects of supplemental short-and medium-chain fatty acids on milk fat synthesis (Modified from Baumgard, L. H., 2002)

In a subsequent study, intestinal availability of SMCFA with BF infusion failed to rescue CLA-induced MFD (Figure 6.2). The transfer efficiency of SCFA was very low, reflecting the trend observed in first study. In addition, SMCFA had no effects on mammary lipogenic gene and protein expression. The results suggest that nutritional manipulation with intestinal SMCFA was insufficient to rescue CLA-induced MFD and that MFD was not solely due to lack of SMCFA precursors.

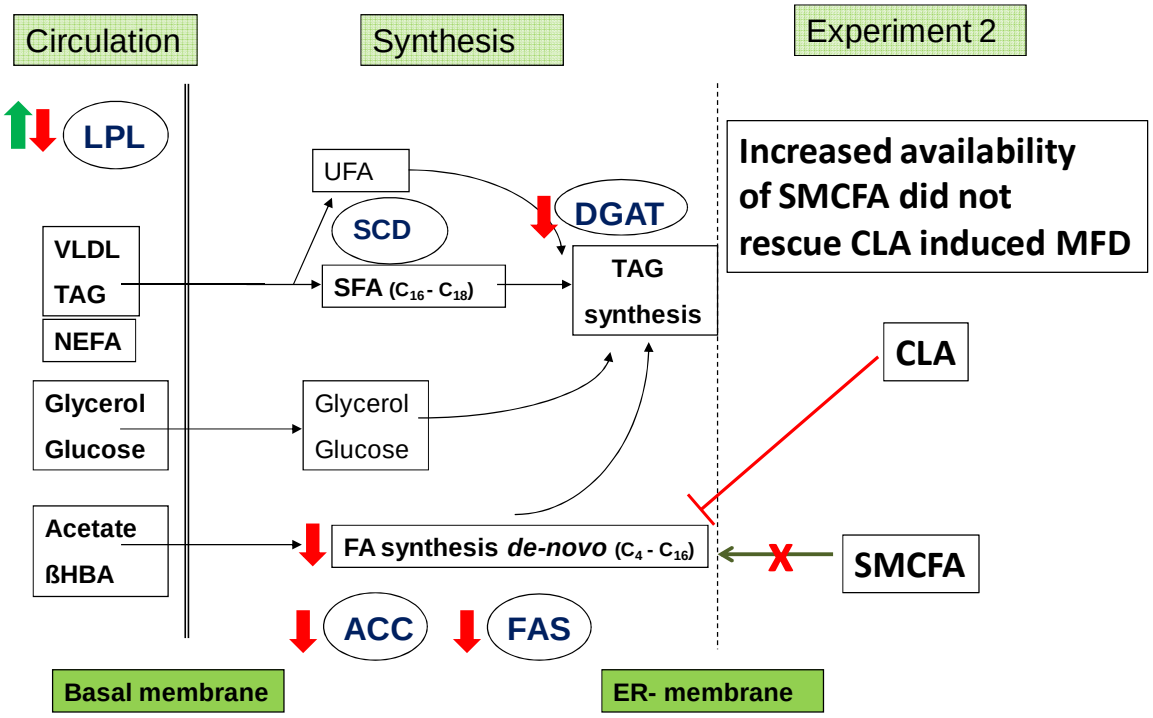


Figure 6.2. The effects of short-and medium-chain fatty acids during conjugated linoleic acid-induced milk fat depression on mammary lipogenesis (Modified from Baumgard L. H., 2002)

Finally, ROSI failed to upregulate mammary lipogenesis and rescue CLA-induced MFD in lactating mice (Figure 6.3). On the contrary, CLA-induced MFD was further increased in presence of ROSI. The results suggested indirect effects of ROSI on mammary gland possibly via increased insulin sensitivity and reducing glucose availability to mammary gland for milk fat synthesis. However, ROSI rescued CLA-induced hepatic steatosis by reducing hepatic lipid accumulation.

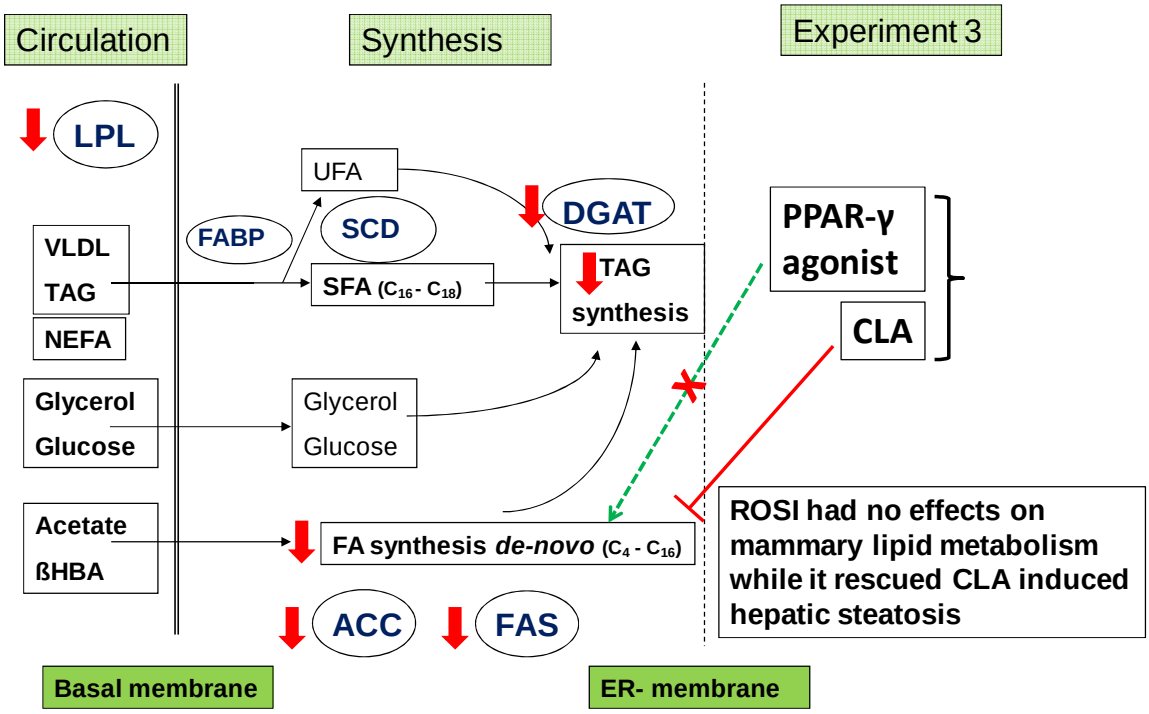


Figure 6.3. The effects of Rosiglitazone, a PPAR-γ agonist, during conjugated linoleic acid-induced milk fat depression on mammary lipogenesis in lactating mice (Modified from Baumgard, L. H., 2002)

To summarize the overall findings, increased availability of nutrient precursors failed to elicit any milk fat responses possibly due to a lack of effect on mammary lipogenic gene and protein expression. Our results further support the role of s*terol regulatory element binding protein-1 (SREBP-1)* as major regulator of mammary lipogenesis while the role of *PPAR- γ* could not be ascertained.

FUTURE DIRECTIONS

Despite advances in our understanding of the role of fatty acids (FA) as regulators of mammary lipogenesis many issues remain unresolved. Previous studies have largely focused on the role of long-chain fatty acids (LCFA) in regulating mammary lipogenesis.

However, future research should be focused on studying the role of individual SMCFA in regulating milk fat synthesis. Because of the limitation of cell culture techniques, most notably the failure to actively secrete milk fat, future studies should either be performed in animal models or freshly isolated tissues capable of secreting milk fat to ensure relevance to the animal's physiology.

Previous studies have demonstrated positive correlation between fat percentage and proportion of SCFA (Palmquist et al, 1993). The transfer efficiency and concentration of SCFA in milk fat was not increased in the present studies despite increasing availability via diet and abomasal infusion. This suggests that future studies should focus on studying the mechanisms regulating the concentration of SCFA in milk fat rather than precursor availability.

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Baumgard, L. H. 2002. Effect of conjugated linoleic acid on lipid metabolism in lactating dairy cows. Thesis (Ph. D.),Cornell University.

Palmquist, D. L., A. D. Beaulieu, and D. M. Barbano. 1993. Feed and animal factors influencing milk fat composition. J. Dairy Sci. 76(6):1753-1771.