Identification of molecular targets regulating fatty acid synthesis in bovine mammary epithelial cells

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ABSTRACT

Consumer demand for milk fat has declined due to the increased risk of cardiovascular disease associated with consuming a high saturated fat diet. Milk fat synthesis is energetically expensive for the dairy cow, especially during early lactation or periods of poor nutrition. Thus, manipulating milk fat production and composition may promote the synthesis of more market-valuable milk components and improve energy utilization in dairy cows during periods of increased energy demand. Therefore, the objective of the present studies was to identify molecular proteins that regulate fatty acid synthesis in bovine mammary epithelial cells. The regulation of lipogenic genes including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) is controlled by transcription factors including sterol regulatory element binding protein-1 (SREBP1) and liver X receptor (LXR). *In vivo*, diet-induced milk fat depression or supplementing diets with polyunsaturated fatty acids inhibits milk fat synthesis by regulating SREBP1 expression. Results confirm that polyunsaturated fatty acids inhibit fatty acid synthesis in bovine mammary epithelial cells by regulating the expression of SREBP1. In hepatocytes, LXR can regulate the transcription of SREBP1 in addition to ACC and FAS. Results confirm that LXR activation enhanced synthesis of fatty acids in bovine mammary epithelial cells by promoting the transcription of FAS and SREBP1. Activation of LXR was unable to prevent the inhibitory effect of polyunsaturated fatty acids on fatty acid synthesis. In the lactating mammary gland, LXR may contribute to the synthesis of fatty acids by

regulating the expression of SREBP1. In addition to modifying the expression of lipogenic genes, some enzymes can be phosphorylated by AMP-activated protein kinase (AMPK), an energy-sensing protein, inhibiting their activity. Presence of AMPK mRNA was identified in bovine mammary epithelial cells and activation of AMPK dramatically decreased fatty acid synthesis in bovine mammary epithelial cells. In the lactating mammary gland, AMPK may sense energy availability and regulate milk fat synthesis to control energy utilization. Identification of SREBP1, LXR, and AMPK as regulators of fatty acid synthesis in bovine mammary epithelial cells may lead to the development of technologies allowing dairy producers to modify milk fat production and composition to meet consumer demand and maximize profitability.

DEDICATION

I dedicate this dissertation to my mother, father, and grandfather.

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ABCG1	ATP-binding cassette transporter-G1
ACC	acetyl-CoA carboxylase
AGPAT	acylglycerol phosphate acyltransferase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMPK	AMP-activated protein kinase
BME-UV	bovine mammary epithelial cell line
BSA	bovine serum albumin
CaMKK	Ca^{2+}/calmodulin-dependent kinase kinase
CD36	cluster of differentiation-36
CLA	conjugated linoleic acid
Cyp1A1	cytochrome P4501A1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
FA	fatty acid
FABP	fatty acid binding protein
FAS	fatty acid synthase
g	gravity
GGPP	geranylgeranyl pyrophosphate
GPAT	glycerol-3-phosphate acyltransferase
INSIG	insulin-induced gene
LA	linoleic acid
LKB1	serine/threonine kinase-11
LPL	lipoprotein lipase
LXR	liver X receptor
LXRE	liver X receptor response element
MAC-T	bovine mammary epithelial cell line
mSREBP	mature sterol regulatory element binding protein
No FA	absence of fatty acids (bovine serum albumin control)
PPAR	peroxisome proliferator-activated receptor
pSREBP	premature sterol regulatory element binding protein
RXR	retinoic X receptor
SCAP	sterol regulatory element binding protein cleavage-activating protein
SRE	sterol response element
SREBP	sterol regulatory element binding protein
TAG	triacylglycerol
T09	T091317
ZMP	5-aminoimidazole-4-carboxamide ribonucleoside monophosphate

CHAPTER 1

General Introduction

Over the past several decades, the Dietary Guidelines for Americans published by the US Department of Health and Human Services and the Department of Agriculture has advocated the consumption of a diet low in total fat, saturated fat, and cholesterol. An increase in the human consumption of milk and butter is associated with an increased risk for the development of cardiovascular disease (Noakes et al., 1996). This increased risk is linked to the ability of saturated fat to elevate the plasma concentration of low density lipoprotein cholesterol which is a primary risk factor for coronary heart disease (LaRosa et al., 1990). Dairy foods provide a significant portion of saturated fat to the human diet which has resulted in a decline in consumer demand for milk fat. Therefore, the development of technologies which modify milk fat production and composition are needed to provide consumers with healthy dairy products.

The utilization of nutritional strategies to manipulate milk fat production and composition appears promising. For decades, producers have observed a decrease in milk fat production in response to feeding high concentrate/low forage diets or dietary supplements of oils high in polyunsaturated fatty acids (Storry and Rook, 1965; Steele et al., 1971). This observation is referred to as milk fat depression (Davis and Brown, 1970; Erdman, 1996; Bauman and Griinari, 2003). Alterations in rumen biohydrogenation of polyunsaturated fatty acids during milk fat depression results in the production of unique fatty acid intermediates which elicit inhibitory effects on milk fat synthesis (Katz and Keeney, 1966; Gaynor et al., 1994). Recently, research has

revealed that specific isomers of conjugated linoleic acid (CLA) are responsible for this inhibitory effect on milk fat production. The most widely studied isomer of CLA to inhibit milk fat synthesis is *trans*-10, *cis*-12 CLA (Baumgard et al., 2000). Development of an *in vitro* approach to study the regulation of fatty acid synthesis in response to *trans*-10, -*cis*-12 CLA is needed to help elucidate the mechanism responsible for this inhibitory effect.

Liver X receptor (LXR) is a nuclear receptor capable of regulating the transcription of sterol regulatory element binding protein-1 (SREBP1) and SREBP1 target genes including acetyl-CoA carboxylase and fatty acid synthase (Schultz et al., 2000; Joseph et al., 2002; Talukdar and Hillgartner, 2006). Sterol regulatory element binding protein-1 is considered to be the primary transcriptional regulator of milk fat synthesis in the bovine mammary gland (Harvatine and Bauman, 2006). Recent evidence demonstrates that the mRNA expression and protein abundance of SREBP1 is decreased in response to *trans*-10, *cis*-12 CLA (Peterson et al., 2004; Harvatine and Bauman, 2006). Whether LXR initiates these effects in the bovine mammary epithelial cell has not yet been documented. Better understanding the role of LXR in controlling lipogenesis may provide researchers with an additional target to prevent diet- or *trans*-10, *cis*-12 CLA-induced decreases in milk fat synthesis.

In addition to modifying milk fat production and composition to meet consumer demand, additional management strategies are needed to maximize animal performance while maintaining animal health. During early lactation, dairy cows often experience enhanced energy demand in response to copious milk secretion, decreased dry matter intake, poor body condition, or sickness. Early lactation cows will mobilize

body lipid reserves as a compensatory mechanism to meet energy demand for milk production, reproductive recovery, and maintenance (Bauman and Davis, 1975). Identification of molecular targets that regulate energy utilization in the mammary gland is necessary to increase milk production or modify milk composition during early lactation or periods of poor nutrition. AMP-activated protein kinase (AMPK) is considered to be the primary gauge of cellular energy supply (see review by Hardie (2007)) and may prove to be a novel target for manipulation in the bovine mammary epithelial cell. Development of strategies that control the activation of AMPK may help alleviate energy deficits during early lactation or poor nutrition ensuring maximum productivity and economic return.

The objective of the following research is to identify various molecular targets that are capable of regulating fatty acid synthesis in bovine mammary epithelial cells. These targets include SREBP1, LXR, and AMPK. Better understanding of these molecular targets and their ability to regulate fatty acid synthesis may lead to the development and utilization of management tools that manipulate milk fat yield and composition based on consumer demand.

CHAPTER 2

Review of Literature

MILK FAT SYNTHESIS

Milk fat is the most variable component of milk and is affected by genetics, physiological state, environment, and nutrition (see review by Palmquist (2006)). This variability can be attributed to the control of mechanisms responsible for the incorporation of over 400 unique fatty acids found in ruminant milk fat (Jensen, 2002). These fatty acids are esterified to form triacylglycerol (TAG), the primary constituent of milk fat. Phospholipids, cholesterol esters, diglycerides, monoglycerides, and free fatty acids are minor contributors to milk fat. The fatty acids found in TAG vary in chain length, degree of unsaturation, and origin. Short-chain fatty acids of 4 to 14 carbons and approximately one-half of palmitate (16 carbons) are synthesized *de novo* from acetate and β-hydroxybutyrate. Preformed long-chain fatty acids greater than 16 carbons and the remainder of palmitate are derived from circulation and make up the remaining fatty acids of TAG. On a molar basis, one-half of milk fatty acids are derived from *de novo* synthesis while the remaining half is derived from circulation (Palmquist et al., 1969; Bauman and Davis, 1974).

De novo fatty acid synthesis

Acetyl-CoA carboxylase (ACC) mediates the first committed step in the synthesis of fatty acids. This lipogenic enzyme is regulated by covalent modification, allosteric mechanisms, and by transcriptional control (Allred and Reilly, 1996; Kim, 1997). Short-term regulation of ACC is achieved by phosphorylation or dephosphorylation.

Phosphorylation of ACC inactivates the enzyme and decreases its activity (Carlson and Kim, 1973). A number of phosphatases are responsible for dephosphorylating ACC thereby promoting its activity (Thampy and Wakil, 1986). Change in the phosphorylation status of ACC occurs within minutes in response to stimuli and are not the result of changes in gene transcription (Merrill et al., 1997). Long-term changes in ACC expression and protein abundance are often observed in response to hormones, nutrients, or other signals (Girard et al., 1994; Baumgard et al., 2002). Multiple transcription factors have been identified as key regulators of the expression of ACC. These include sterol regulatory element binding protein-1 (SREBP1), liver X receptor-α (LXRα), and peroxisome proliferator-activated receptor-γ (PPARγ), further described below.

Fatty acid synthase (FAS) is responsible for synthesizing saturated fatty acids ranging from 4 to 16 carbons in length from acetyl-CoA, malonyl-CoA, and nicotinamide adenine dinucleotide phosphate. Similarly to ACC, FAS is regulated by various lipogenic transcription factors that promote the transcription of FAS in response to appropriate hormonal or nutrient signals (Magana and Osborne, 1996; Joseph et al., 2002). Sterol regulatory element binding protein-1, LXRα, and PPARγ are examples of such transcription factors detailed below.

Uptake of preformed fatty acids

Long-chain fatty acids found in milk fat are derived from nonesterified fatty acids, TAG-rich chylomicra, and very low density lipoproteins found in circulation. In lactating dairy cows, circulating concentrations of nonesterified fatty acids are highly correlated

with rate of lipolysis (Bauman et al., 1988). Thus the incorporation of long-chain fatty acids is greatest during early lactation in response to the onset of negative energy balance and the associated release of nonesterified fatty acids from adipose (Palmquist et al., 1993). The hydrolysis of TAG to free fatty acids and glycerol is mediated by mammary lipoprotein lipase (LPL). Activity of mammary LPL is increased immediately prior to parturition and remains elevated throughout lactation (Shirley et al., 1973; Liesman et al., 1988). The expression of LPL has been shown to be tightly controlled by hormonal and nutritional signals (Liesman et al., 1988; Baumgard et al., 2002).

Mechanisms responsible for the transport of fatty acids from capillaries to the interior of the mammary epithelial cell are still being defined. Barber and coworkers (1997) have proposed the involvement of cluster of differentiation-36 (CD36), a fatty acid translocator, in conjunction with intracellular fatty acid binding protein (FABP) to transport fatty acids across the cell membrane. Multiple FABPs have been implicated in transmembrane and intracellular transport of fatty acids (Hertzel and Bernlohr, 2000).

Prior to utilization, long-chain fatty acids are esterified with CoA in the inner surface of the plasma membrane. Acyl-CoA synthetase has been shown to be responsible for this activation (Mashek and Coleman, 2006). Lastly, acyl-CoA-binding proteins may also play an integral role in the transport of fatty acids (Knudsen et al., 2000). These binding proteins have been shown to bind long-chain acyl-CoA much more effectively than FABP (Rasmussen et al., 1990). Their definitive role in fatty acid transport needs further exploration.

Triacylglycerol synthesis

Triacylglycerol synthesis requires the actions of multiple enzymes to esterify three fatty acids to glycerol (see review by Coleman and Lee (2004)). Glycerol-3-phosphate required for esterification of fatty acids is generated by glycolysis or by phosphorylation of free glycerol by glycerol kinase (Bickerstaffe and Annison, 1971). Glycerol-3-phosphate acyltransferase (GPAT) is the first enzyme involved in TAG synthesis producing the first of three acylations at *sn*-1 producing lysophosphatidate. Palmitoyl-CoA has been shown to be the preferred substrate for the initial acylation of glycerol-3-phosphate in bovine mammary tissue (Kinsella and Gross, 1973). Acylglycerol-3-phosphate acyltransferase (AGPAT) is responsible for converting lysophosphatidate to phosphatidate while phosphatidic acid phosphatase is responsible for the second acylation at *sn*-2. Lastly, diacylglycerol acyltransferase is responsible for the final acylation forming TAG.

Coordinate regulation of lipogenic enzyme expression

Expression and activity of lipogenic enzymes and utilization of substrates by mammary tissue increases dramatically at the onset of lactation (Wilde et al., 1986; Bauman and Currie, 1980; Bionaz and Loor, 2008b). For instance, the mRNA abundance at 60 d postpartum for FABP3, acyl-CoA synthetase, and AGPAT6 was 80-, 7-, and 15-fold greater relative to 15 d prior to parturition (Bionaz and Loor, 2008a). Interestingly, the expression pattern of enzymes involved in *de novo* fatty acid synthesis, TAG synthesis, and fatty acid transport are similar to that of a lactation curve with highest expression at peak milk production. Bionaz and Loor (2008b) observed

peak expression of ACC, FAS, LPL, GPAT, AGPAT6, and diacylglycerol acyltransferase-1 at 60 days in milk. Similarly, the mRNA expression of CD36, FABP3, acyl-CoA synthetase-1, and acyl-CoA-binding protein mimicked these results (Bionaz and Loor, 2008b). The regulation of lipogenic enzyme expression is thought to be controlled by central regulators of transcription (Schultz et al., 2000; Gerhold et al., 2002; Stoeckman and Towle, 2002). These regulators are transcription factors that control the expression of lipogenic genes. Three recognized transcription factors that may control lipid synthesis in the mammary gland are SREBP1, LXRα, and PPARγ.

LIPOGENIC TRANSCRIPTION FACTORS

Transcription factors are cellular proteins capable of regulating gene expression by binding to specific DNA sequences found within the promoter region of target genes. Select transcription factors are referred to as nuclear receptors. Nuclear receptors must bind to specific ligands in the nucleus in order to promote transcription. Example ligands include hormones, prostaglandins, fatty acids, bile acids, and oxysterols. Unlike SREBPs, LXRs and PPARs are nuclear receptors. Sterol regulatory element binding proteins, LXRs, and PPARs are three highly characterized transcription factors known to regulate the synthesis of fatty acids.

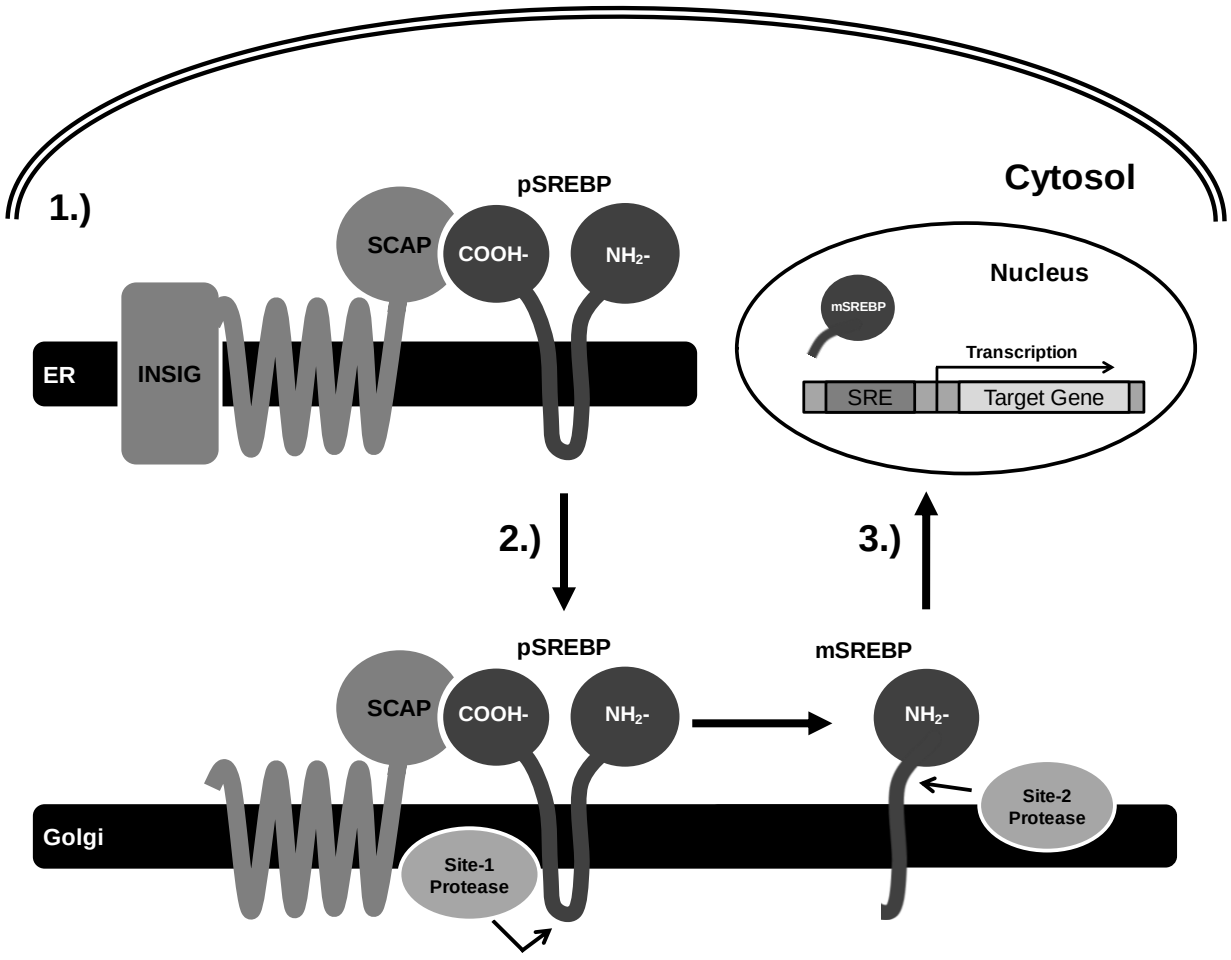
Sterol regulatory element binding proteins

Three known isoforms of SREBP currently exist in the mammalian genome. Sterol regulatory element binding protein-1a and SREBP1c are derived from a single gene through the use of alternate transcription start sites (Hua et al., 1995; Shimomura

et al., 1997). Sterol regulatory element binding protein-1a is the primary isoform expressed in cultured cells while in most animal tissues, SREBP1c is the predominant isoform (Shimomura et al., 1997). Sterol regulatory element binding protein-1a can activate all SREBP-responsive genes while the synthesis of fatty acids is the predominant role of SREBP1c (Horton et al., 2002). The third isoform, known as SREBP2, is derived from a different gene and is involved in the transcriptional regulation of cholesterol homeostasis (Hua et al., 1995).

In order to promote transcription of lipogenic enzymes, SREBP must be proteolytically cleaved (see reviews by Brown and Goldstein (1997) and McPherson and Gauthier (2004); see Figure 2.1). Newly synthesized SREBP, referred to as premature SREBP (pSREBP), is embedded in the membrane of the endoplasmic reticulum. In order for SREBP to initiate transcription, the NH_2-terminal domain must be released from the membrane. The COOH-terminal regulatory domain binds to the COOH-terminal domain of SREBP cleavage-activating protein (SCAP). Insulin induced gene-1 (INSIG1) and INSIG2 are integral membrane proteins that bind SCAP and cause retention of SREBP in the endoplasmic reticulum. When not bound to INSIG, SCAP escorts SREBP from the endoplasmic reticulum to the golgi apparatus where two proteases, known as Site-1 protease and Site-2 protease, are present. These proteases are responsible for cleaving SREBP causing the release of the mature, active form of SREBP (mSREBP). The mature form of SREBP translocates to the nucleus and binds to sterol response elements (SRE) found within the promoter regions of target genes thereby promoting transcription.

Figure 2.1. Proteolytic cleavage of sterol regulatory element binding protein (SREBP). (1) Newly synthesized sterol regulatory element binding protein (SREBP), referred to as premature SREBP (pSREBP), is embedded in the membrane of the endoplasmic reticulum. In order for SREBP to initiate transcription, the NH_2-terminal domain must be released from the membrane. The COOH-terminal regulatory domain binds to the COOH-terminal domain of SREBP cleavage-activating protein (SCAP). Insulin-induced genes (INSIG) are integral membrane proteins that bind SCAP and cause retention of SREBP in the endoplasmic reticulum. (2) When not bound to INSIG, SCAP escorts SREBP from the endoplasmic reticulum to the golgi apparatus where two proteases, known as Site-1 protease and Site-2 protease, are present. These proteases are responsible for cleaving SREBP releasing the mature, active form of SREBP (mSREBP). (3) The mature form of SREBP translocates to the nucleus and binds to sterol response elements (SRE) found within the promoter regions of target genes thereby promoting transcription. Modified from DeBose-Boyd (2008).



Sterol regulatory element binding proteins can regulate fatty acid synthesis by controlling the transcription of lipogenic enzymes (Kim et al., 1998; Stoeckman and Towle, 2002). The promoters of the lipogenic enzymes ACC, FAS, and GPAT all contain a SRE capable of binding SREBP1 (Lopez et al., 1996; Magana and Osborne, 1996; Ericsson et al., 1997). The binding of SREBP1 to these promoters increases lipid synthesis by influencing transcription. In rat hepatocytes, SREBP1c is necessary for the stimulation of ACC and FAS by glucose (Foretz et al., 1999). Overexpression of SREBP1c in adipocyte cell lines increases FAS expression (Kim et al., 1998) and overexpression of mSREBP1c in the liver of transgenic mice increases liver TAG (Shimano et al., 1997). Sterol regulatory element binding protein-1c regulates TAG synthesis by controlling the transcription of GPAT in 3T3-L1 preadipocytes (Ericsson et al., 1997).

Liver X receptors

Liver X receptors are nuclear receptors that regulate the synthesis of lipid including fatty acids, cholesterol, and bile acids (see review by Edwards et al. (2002)). Liver X receptors are regulated by oxysterols that appear to be produced in proportion to cellular cholesterol content (Janowski et al., 1999). Liver X receptor-α and LXRβ are two known isoforms of LXR. Liver X receptor-α is expressed in adipose, liver, and intestine while LXRβ is expressed ubiquitously (Peet et al., 1998a). These nuclear receptors regulate the transcription of target genes by binding to DNA in a heterodimeric complex with retinoic X receptor (RXR; Willy et al., 1995).

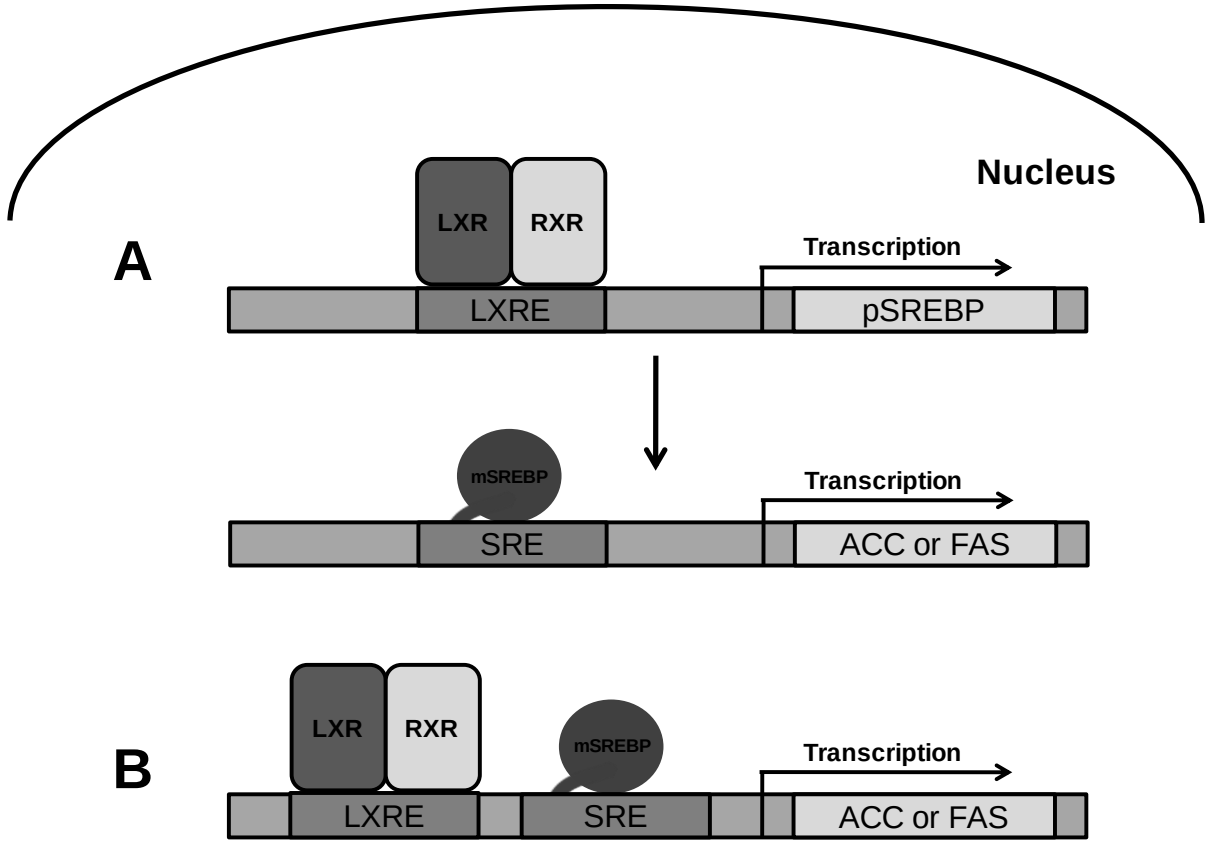
Liver X receptors regulate fatty acid synthesis by increasing the transcription of lipogenic enzymes via indirect and direct mechanisms (see Figure 2.2). Transcription of SREBP1 mRNA in liver and intestine was increased in response to T091317 (T09), a LXR agonist (Repa et al., 2000; Schultz et al., 2000). This response failed to occur in LXRα/LXRβ double knockout mice (Repa et al., 2000). In support, T09 has been shown to increase the concentration of mSREBP in the nucleus (Talukdar and Hillgartner, 2006; Montanaro et al., 2007). The ability of T09 to promote the transcription of SREBP is specific for the SREBP1c isoform (Repa et al., 2000; Schultz et al., 2000). As mentioned above, LXR must form a heterodimer with RXR in order to regulate the transcription of target genes. Co-transfection of LXR with RXR synergistically activates the SREBP1c promoter in HePG2 cells (Yoshikawa et al., 2001).

In addition to increasing the transcription of SREBP1c, LXR can directly promote the transcription of target genes by binding to a response element found within the promoter region. Joseph and coworkers (2002) demonstrated that in addition to tandem SREBP1c sites, the FAS promoter contains a binding site for the LXR/RXR heterodimers. They conferred that maximum induction of FAS requires both LXR and SREBP1c binding to their respective response elements within the promoter region. Similarly, Talukdar and Hillgartner (2006) demonstrated that LXR increases ACCα transcription by activating LXR/RXR heterodimers bound to a LXR response element (LXRE).

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors are nuclear receptor proteins capable

Figure 2.2. Liver X receptors (LXR) regulate fatty acid synthesis by increasing the transcription of lipogenic enzymes via indirect and direct mechanisms. (A) LXR can activate the transcription of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) indirectly by increasing the transcription of premature sterol regulatory element binding protein (pSREBP). Liver X receptor forms a heterodimer with retinoic X receptor (RXR) and binds to the LXR response element (LXRE) located within the promoter region of pSREBP. Mature SREBP can then bind to the sterol response element (SRE) further promoting the transcription of ACC and FAS. (B) In addition to increasing the transcription of pSREBP, LXR can directly promote the transcription of ACC and FAS by binding to the LXRE found within the promoter region of these genes.



of regulating cellular differentiation and metabolism of lipid (see review by Berger and Moller (2002)). As is observed with several nuclear receptors, such as LXR, PPARs must form a heterodimer with RXR for DNA binding. Three related PPAR isoforms, encoded by separate genes, have been identified. These include PPARα, PPARδ, and PPARγ. Peroxisome proliferator-activated receptors are ligand-activated transcription factors capable of regulating gene expression by binding to specific peroxisome proliferator response elements found within the promoter region of target genes. Such ligands include fatty acids, eicosanoids, and synthetic antidiabetic thiazolidinediones (Krey et al., 1997).

Peroxisome proliferator-activated receptor-α is primarily expressed in tissues with elevated rates of fatty acid oxidation such as liver, heart, brown adipose, and skeletal muscle (Braissant et al., 1996; Auboeuf et al., 1997). Peroxisome proliferator-activated receptor-α ligand binding has profound effects on fatty acid oxidation and lipoprotein metabolism. Enzymes involved in fatty acid oxidation such as medium chain acyl-CoA dehydrogenase, acyl-CoA oxidase, and cytochrome P450 fatty acid ω-hydroxylase are upregulated by PPARα (Schoonjans et al., 1996b). Furthermore, carnitine palmitoyltransferase-I has been shown to be upregulated by PPARα via a response element found within the promoter region of the gene (Mascaro et al., 1998). In addition, PPARα knockout mice failed to increase the expression of genes involved in fatty acid oxidation in response to stimulation with PPARα agonists (Lee et al., 1995). As a result of the shift in free fatty acid metabolism from TAG synthesis to catabolism in response to PPARα agonists, the secretion of very low density lipoproteins is strongly decreased (Schoonjans et al., 1996b).

Peroxisome proliferator-activated receptor-δ is expressed ubiquitously in white adipose, skeletal muscle, heart, and intestinal tissue (Braissant et al., 1996). Current evidence suggests that PPARδ may play a role in the metabolism of lipids and the pathophysiology of atherosclerosis. Treatment of insulin-resistant obese rhesus monkeys with a PPARδ agonist decreased blood TAG, fasting plasma insulin, and low density lipoprotein cholesterol (Oliver et al., 2001). Treatment of myotubes with a PPARδ agonist increased fatty acid oxidation (Tanaka et al., 2003). In addition, ligand binding to PPARδ results in decreased expression of inflammatory cytokine genes and diminished inflammation (Lee et al., 2003).

Peroxisome proliferator-activated receptor-γ is predominantly found in white adipose tissue (Braissant et al., 1996). Peroxisome proliferator-activated receptor-γ is the most widely characterized PPAR isoform and has been shown to play a central role in the control of adipocyte gene expression and differentiation (Brun et al., 1996). Direct activation of PPARγ induces adipocyte genes such as LPL, fatty acid transport protein, and CD36, enzymes essential for the cellular uptake of lipid (Schoonjans et al., 1996b; Martin et al., 1997; Sfeir et al., 1997). In addition, liver PPARγ ablation significantly decreases ACC and FAS mRNA in mice (Gavrilova et al., 2003). Interestingly, coexpression of PPARγ with SREBP has been shown to increase the transcriptional activity of PPARγ (Kim et al., 1996).

Transcription factors in the mammary gland

The molecular regulation of milk fat synthesis in the bovine mammary gland involves multiple regulatory pathways. Recent research has helped determine the

relative expression of SREBPs, LXRs, and PPARs during the dry period and lactation (Bionaz and Loor, 2008a, Bionaz and Loor, 2008b); however, further research is needed to help identify the relative contributions of each transcription factor to the synthesis of milk fat.

Sterol regulatory element binding protein is considered to be the primary regulator of milk fat synthesis in the lactating mammary gland (Harvatine and Bauman, 2006). The expression of SREBP1 is significantly upregulated during lactation in mice and cows (Anderson et al., 2007; Rudolph et al., 2007; Bionaz and Loor, 2008b). *Trans*-10, *cis*-12 CLA significantly decreases the proteolytic activation of SREBP1 in MAC-T (Peterson et al., 2004). In support, Harvatine and Bauman (2006) observed a downregulation of SREBP1 and SREBP1-regulated enzymes during diet-induced milk fat depression in the lactating dairy cow. Expression of SREBP regulatory proteins (INSIG1, INSIG2, and SCAP) is significantly increased throughout the first 120 d of lactation in the dairy cow (Bionaz and Loor, 2008b). In addition, Harvatine and Bauman (2006) observed a significant decrease in INSIG1 and INSIG2 mRNA in response to *trans*-10, *cis*-12 CLA further implicating SREBP1 in the synthesis of milk fat.

The function of LXR in the mammary gland is undefined. In mice, the expression of LXRα in the mammary gland is approximately 10-fold greater during early pregnancy compared to early lactation (Anderson et al., 2007). In addition to LXRα, LXRβ has been shown to be present in appreciable amounts in the lactating mammary gland of mice (Rudolph et al., 2007). In dairy cows, LXRα, but not LXRβ expression is increased during lactation compared to nonlactating mammary tissue (Harvatine and Bauman, 2007; Bionaz and Loor, 2008b). In contrast, Farke and coworkers (2008) observed no

difference in LXRα mRNA expression between dry period and lactation in dairy cows. Harvatine and Bauman (2007) determined that the expression of LXRα and LXRβ are not modified during milk fat depression induced by diet or treatment with *trans*-10, *cis*-12 CLA; however the activity was not assessed.

Similarly to LXR, the role of PPARγ in the mammary gland still remains to be defined. In mice, the expression of PPARγ is approximately 10-fold greater during early pregnancy compared to early lactation (Anderson et al., 2007). In contrast, Bionaz and Loor (2008b) observed a significant increase in the mRNA expression of PPARγ during lactation. Bovine mammary epithelial cells treated with rosiglitazone, a PPARγ agonist, resulted in a significant upregulation of FAS and SREBP1 expression (Kadegowda et al., 2008). The observed increase in SREBP1 expression may be explained by an increase in PPARγ coactivator-1A expression throughout the first 120 d of lactation (Bionaz and Loor, 2008b). The observed increase in PPARγ coactivator-1A was accompanied by a parallel increase in INSIG1. Interestingly, INSIG1 has been shown to be a PPARγ responsive gene (Kast-Woelbern et al., 2004; Kadegowda et al., 2008). Therefore, PPARγ may play a role in the regulation of SREBP1. Lastly, unlike SREBP1, the expression of PPARγ is not modified by milk fat depression in lactating dairy cows (Harvatine and Bauman, 2007). Whether or not the activity of PPARγ is modified by milk fat depression remains undefined.

REGULATION OF MILK FAT SYNTHESIS BY FATTY ACIDS

During the past decade, a plethora of research has focused on the regulation of milk fat synthesis by CLAs (see review by Bauman and coworkers (2008)). Conjugated

linoleic acids are produced as intermediates in the biohydrogenation of linoleic acid (LA) by rumen microbes (Davis and Brown, 1970). *Cis*-9, *trans*-11 CLA is the predominant CLA isomer found in milk fat (Parodi, 1997). This isomer is derived primarily from the desaturation of *trans*-vaccenic acid within the mammary gland (Griinari et al., 2000). In addition to *cis*-9, *trans*-11 CLA, a variety of other CLA isomers are detected in milk fat including *trans*-10, *cis*-12 CLA (Piperova et al., 2002). In 2000, Baumgard and coworkers (2000) identified *trans*-10, *cis*-12 CLA as the unique CLA isomer responsible for dramatic reductions in milk fat synthesis. They also observed that *cis*-9, *trans*-11 CLA had no effect on milk fat content or yield. In addition to the well documented effects of *trans*-10, *cis*-12 CLA on milk fat synthesis (Baumgard et al., 2000; Baumgard et al., 2002), *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA have been identified as inhibitors of milk fat synthesis (Saebo et al., 2005; Perfield et al., 2007).

Suppression of lipogenic enzymes by trans-10, cis-12 conjugated linoleic acid

The effects of *trans*-10, *cis*-12 CLA on lipogenic gene expression in the bovine mammary gland have been well established. Abomasal infusions of *trans*-10, *cis*-12 CLA have resulted in ~40-50% reductions in ACC, FAS, GPAT, AGPAT, and LPL mRNA expression (Baumgard et al., 2002). Sterol regulatory element binding protein-1 is a known regulator of these enzymes; therefore, it has been proposed that SREBP1 may be responsible for regulating their expression in response to *trans*-10, *cis*-12 CLA (Peterson et al., 2004; Harvatine et al., 2009). In support of this theory, the mRNA expression of SREBP1 is significantly decreased in response to *trans*-10, *cis*-12 CLA treatment (Harvatine and Bauman, 2006). Interestingly, the mRNA expression of

INSIG1 was also decreased in response to treatment. Expression of PPARα, PPARβ, PPARγ, LXRα and LXRβ was not modified by intravenous infusion of *trans*-10, *cis*-12 CLA (Harvatine and Bauman, 2007). Therefore, decreases in lipogenic gene expression in response to *trans*-10, *cis*-12 CLA are potentially due to a decrease in SREBP1 gene expression rather than changes in nuclear receptor gene expression.

The ability of specific CLA isomers to inhibit milk fat synthesis seems to be unique to these polyunsaturated fatty acids. Saturated fatty acids have been shown to have no effect on milk fat production (Drackley et al., 1992; Bremmer et al., 1998). In addition, oleic acid, a monounsaturated fatty acid, fails to inhibit milk fat production. For instance, LaCount and coworkers (1994) observed an increase in milk fat content and yield in response to abomasal infusions of either canola oil (high in oleic acid and LA) or a high oleic acid, sunflower oil. In addition, abomasal infusion of oils high in *trans*-fatty acids significantly decreased milk fat production in dairy cows compared to a high oleic acid, sunflower oil infusion (Gaynor et al., 1994). Lastly, LA alone fails to inhibit milk fat production in the absence of CLAs (Loor and Herbein, 1998).

In vitro studies

The *in vitro* study of the regulation of fat synthesis by fatty acids varying in degree of saturation has been challenging. Few studies have evaluated the effect of fatty acids varying in degree of saturation on lipid synthesis in bovine mammary epithelial cells. In bovine mammary epithelial cells, addition of palmitic acid to the incubation medium stimulated synthesis of butyric acid and palmitic acid (Hansen and Knudsen, 1987). Contrary to *in vivo* data, Hansen and Knudsen (1987) also observed

that the effect of oleic acid addition on the synthesis and esterification of short- and medium-chain fatty acids was strongly inhibitory. These results were also observed in goat mammary epithelial cells (Hansen et al., 1984).

Recently, a select number of studies have evaluated the effect of CLA isomers on milk fat synthesis *in vitro* yielding confounding results. Incubation of MAC-T cells with increasing concentrations (20-100 µM) of oleic acid and *trans*-vaccenic acid decreased the activities of ACC and FAS (Jayan and Herbein, 2000). Treatment of MAC-T with 75 µM *trans*-10, *cis*-12 CLA resulted in a 50% reduction in the incorporation of radiolabeled acetate (Peterson et al., 2004). However, incubation of MAC-T with 75 µM *cis*-9, *trans*-11 CLA failed to significantly decrease the mRNA expression of ACC and FAS compared to *trans*-10, *cis*-12 CLA. Contrary to Harvatine and Bauman (2006), Peterson and coworkers (2004) observed no significant difference in the mRNA expression of SREBP1 between *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA treatments compared to a bovine serum albumin control. When comparing *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, *trans*-10, *cis*-12 CLA increased pSREBP protein and decreased mSREBP compared to *cis*-9, *trans*-11 CLA (Peterson et al., 2004).

The potential differences between *in vitro* and *in vivo* data may be due to nonspecific effects of incubating bovine mammary epithelial cells with free fatty acids. For instance, Keating and colleagues (2008) observed that increasing concentrations of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA have a negative impact on MAC-T cell growth. Decreases in cell growth may be responsible for observed decreases in mRNA expression and protein abundance of lipogenic genes. It may also be possible that the *in vitro* inhibition of lipogenic enzyme activity by fatty acids varying in degree of

saturation may occur in response to a nonspecific detergent effect. Pande and Meade (1968) state that fatty acids or acyl-CoA esters acting as potent detergents can bind to proteins and affect their secondary and tertiary structure potentially altering their biological activity. This theory was later supported by Bauman and Davis (1974). To answer this question, Wright and coworkers (2002) incubated bovine mammary explants with either palmitic acid or sodium dodecyl sulfate. They observed that palmitic acid inhibited fatty acid synthesis and that this effect was not the result of a nonspecific detergent effect. To study the effects of *trans*-10, *cis*-12 CLA in cell culture, further research is needed to identify the differences between *in vitro* and *in vivo* data.

AMP-ACTIVATED PROTEIN KINASE

AMP-activated protein kinase is critical in ensuring the energy balance of mammalian cells (see review by Hardie (2007)). Mammalian AMPK is highly sensitive to increases in the AMP:ATP ratio and is activated by metabolic stresses that inhibit ATP production or stimulate ATP consumption. Most notably, AMPK is activated in response to exercise, fasting, and hypoxia (Mu et al., 2001; Minokoshi et al., 2004). It is also modulated by cytokines that regulate energy balance (i.e. leptin; Minokoshi et al., 2002), pharmacological activators (i.e. 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR); Corton et al., 1995), or natural plant products (i.e. (-)-epigallocatechin-3-gallate; Collins et al., 2007). 5-Aminoimidazole-4-carboxamide ribonucleoside may be phosphorylated to form AICAR monophosphate (ZMP), the monophosphorylated derivative of AICAR (Corton et al., 1995). 5-Aminoimidazole-4-carboxamide ribonucleoside monophosphate mimics the effects of AMP on AMPK by causing

allosteric activation but also promotes the phosphorylation and activation of an upstream AMPK kinase (Corton et al., 1995; Henin et al., 1995). Once activated, AMPK is able to stimulate ATP production in addition to inhibiting ATP consumption by enhancing the uptake and metabolism of glucose and fatty acids as well as inhibit the synthesis of fatty acids, cholesterol, glycogen, and protein (Kahn et al., 2005). Switching on catabolic pathways and inhibiting anabolic pathways ensures a decrease in the AMP:ATP ratio thus restoring energy homeostasis of the cell.

Structure of AMP-activated protein kinase

AMP-activated protein kinase is a complex heterotrimeric protein consisting of one catalytic subunit (α) and two regulatory subunits (β and γ). In mammalian cells, multiple isoforms exist for the α (α1, α2), β (β1, β2), and γ (γ1, γ2, and γ3) subunits of AMPK. Each subunit plays a distinct role in the overall function of AMPK. The γ subunit of AMPK binds AMP causing a conformational change exposing the catalytic domain found on the α subunit. Exposing the AMP binding domain on the α subunit results in the phosphorylation of threonine-172 by upstream AMPK kinases (Hawley et al., 1996). The β subunit tethers the α and γ subunits of AMPK (Iseli et al., 2005). In addition, the β subunit contains a carbohydrate binding domain that is capable of binding glycogen causing a conformational change in the structure of the protein (Hudson et al., 2003).

All three subunits are required to yield significant AMPK activity (Dyck et al., 1996). At least twelve heterotrimeric combinations are possible (i.e. α1, β1, γ1 or α1, β2, γ3) and can vary based on specie and tissue specificity. The tissue-specific

distribution of AMPK subunits in different species may explain differences in metabolic response to AMPK activation (Chen et al., 1999; Cheung et al., 2000; Birk and Wojtaszewski, 2006). As an example, the predominant AMPK heterotrimeric complex in skeletal muscle contains the α2, β2, γ3 isoforms (Barnes et al., 2004; Mahlapuu et al., 2004).

AMP-activated protein kinase is highly expressed in skeletal muscle, liver, heart, and mammary gland of rats (Verhoeven et al., 1995; Woods et al., 1996). It should be noted that expression does not always correlate with activity of AMPK. The α1 isoform accounts for 90% of total AMPK activity in liver extracts yet the mRNA level of α1 was found to be low compared to the expression of the α2 isoform (Gao et al., 1996). Verhoeven and coworkers (1995) showed that total AMPK mRNA was 4 fold greater in skeletal muscle compared to heart, liver, mammary gland, and brain; however, activity failed to mimic expression. Cheung and others (2000) observed that AMPK activity in rats was greatest in liver, lung, and heart and lower in muscle, testis, brain, and pancreas. Similarly, AMPK activity is greatest in the kidney, liver, and heart of rat in response to AMP (Stapleton et al., 1996).

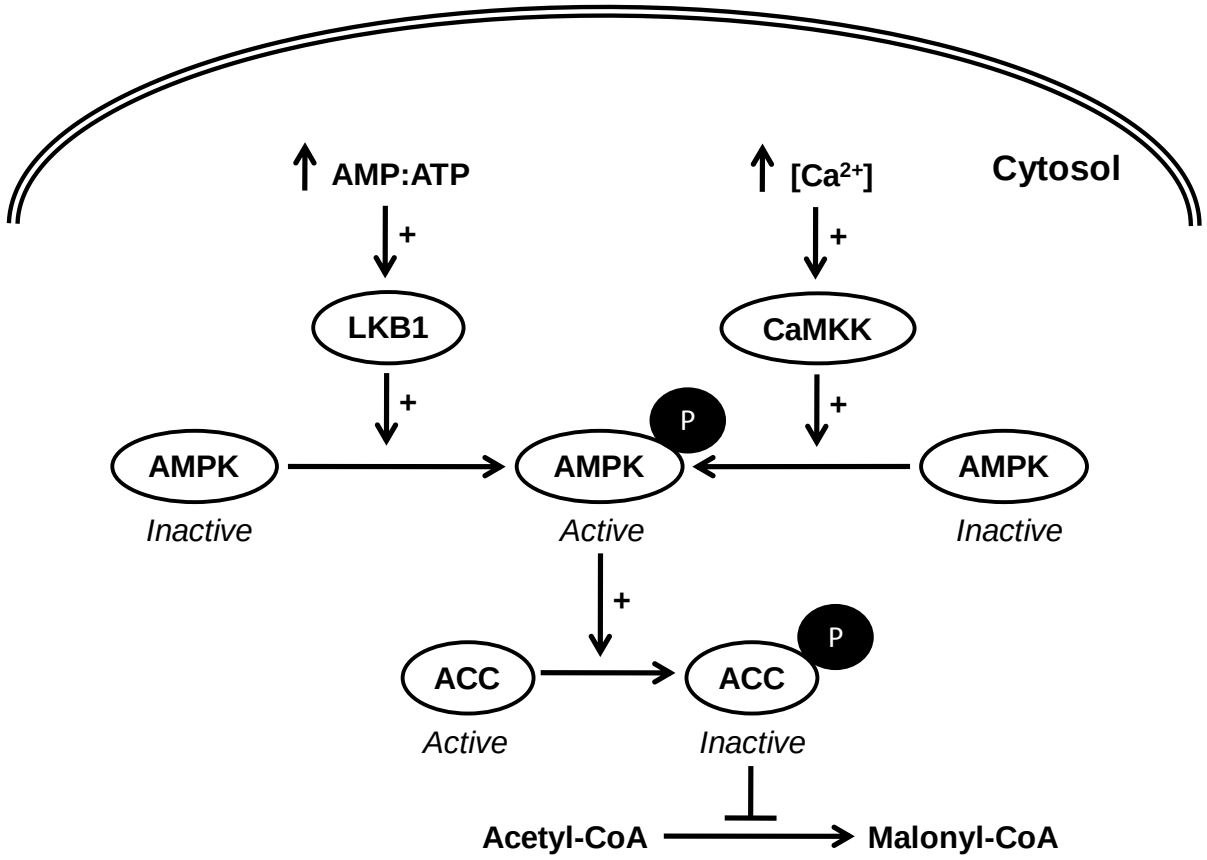
Regulation by upstream kinases

LKB1 is a serine/threonine kinase and is the predominant AMPK kinase in multiple mammalian cell types in response to changes in the AMP:ATP ratio (Hawley et al., 2003; Shaw et al., 2004). As mentioned, AMP binds to the γ subunit of AMPK allowing for a conformational change exposing the catalytic domain found on the α subunit. LKB1 can activate AMPK by directly phosphorylating α1 and α2 subunits of

AMPK in addition to eleven other AMPK-related kinases (Lizcano et al., 2004). Specifically, this upstream AMPK kinase directly phosphorylates threonine-172 of α subunit of AMPK thereby activating AMPK (Shaw et al., 2004). Activation of AMPK by LKB1 can result in the phosphorylation (inactivation) of ACC thereby inhibiting lipid synthesis (Shaw et al., 2004). Sakamoto and coworkers (2005) used mice with decreased expression of muscle LKB1 and found that phosphorylation of ACC was profoundly reduced. Lastly, studies have shown that phenformin and AICAR fail to activate AMPK in cells deficient in LKB1 (Hawley et al., 2003).

Ca^{2+}/calmodulin-dependent kinase kinase is another upstream kinase responsible for activating AMPK (Figure 2.3). Contrary to LKB1, CaMKK activates AMPK in an AMP-independent manner by recognizing increasing concentrations of intracellular Ca^{2+} (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). Therefore, in addition to fluctuations in the AMP:ATP ratio, AMPK may be responsive to nutrients, changes in physiological conditions, hormonal stimuli, or pharmaceutical drugs. For instance, α-lipoic acid and (-)-epigallocatechin-3-gallate, two natural compounds, activate CaMKK in myotubes and hepatocytes, respectively (Collins et al., 2007; Shen et al., 2007). Ionomycin, an ionophore responsible for increasing intracellular Ca^{2+}, has been shown to increase AMPK activity and ACC phosphorylation in HeLa cells (Hurley et al., 2005). Similarly, Woods and others (2005) observed an increase in AMPK activity with ionomycin treatment in HeLa cells. Co-incubating ionomycin with STO-609, a cell permeable inhibitor of CaMKK, resulted in a reduction in ionomycin induced AMPK activity (Woods et al., 2005) and is supported by Hurley and coworkers (2005). Lastly, ionomycin stimulated AMPK activity and ACC

Figure 2.3. Phosphorylation (activation) of AMP-activated protein kinase (AMPK) by serine/threonine kinase-11 (LKB1) and Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) results in the phosphorylation (inactivation) of acetyl-CoA carboxylase (ACC).



phosphorylation are decreased in response to downregulating CaMKKβ, one of two isoforms of CaMKK, using RNA interference (Hurley et al., 2005; Woods et al., 2005).

Regulation of acetyl-CoA carboxylase

Phosphorylation of ACC *in vitro* and *in vivo* has been well documented and currently two known protein kinases significantly inactivate this lipogenic enzyme – cAMP-dependent protein kinase A and AMPK (Munday et al., 1988). Regulation of ACC by AMPK is shown in Figure 2.3. In rat, ACCα is phosphorylated by AMPK on serine residues 79, 1200, and 1215 while cAMP-dependent protein kinase A phosphorylates serine residues 77 and 1200. Phosphorylation of ACC by cAMP-dependent protein kinase A and AMPK produces 10% and 90% decreases in the V_{max} of the enzyme, respectively (Munday et al., 1988). Removal of the N-terminus of ACCα results in the re-activation of the cAMP-dependent protein kinase A or AMPK mediated phosphorylation indicating that only serine residues 77 and 79 exhibit the inhibitory effect on ACCα (Davies et al., 1990). Finally, mutation of serine-79 on ACCα in HeLa cells abolishes the inhibitory effect of AMPK phosphorylation and prevents serine-1200 phosphorylation (Ha et al., 1994). *In vitro,* ACCα and ACCβ in rat liver can both serve as a substrate for AMPK phosphorylation causing inactivation (Dyck et al., 1996; Winder et al., 1997).

Metabolic stress can result in the phosphorylation of ACC by AMPK; thereby inhibiting lipid synthesis. Rats running on a treadmill resulted in a 2.4 fold increase in activation of AMPK in muscle and a concurrent 67% decrease in ACC activity in muscle (Winder and Hardie, 1996). In agreement, Park and coworkers (2002a) observed a

50% decrease in ACC activity in the muscle and liver of exercised rats. Park and others (2002b) showed a significant negative correlation between AMPK-phosphorylated ACC and ACC activity. In bovine aortic endothelial cells, hypoxia caused an increase in AMPK and ACC phosphorylation (Zou et al., 2003). Mimicking the effects of increased energy demand has also been shown to inactivate ACC in response to AMPK activation (Merrill et al., 1997; Zhou et al., 2001; Park et al., 2002a).

Regulation of glycerol-3-phosphate acyltransferase

AMP-activated protein kinase may downregulate TAG synthesis to promote the partitioning of fatty acyl-CoA towards β-oxidation (Muoio et al., 1999). In oxidative muscle isolated from mice, incorporation of [^{14}C]-oleate into TAG decreased by 37% in response to AICAR (Muoio et al., 1999). Likewise incubating cultured rat hepatocytes with AICAR decreased [^{14}C]-oleate incorporation into TAG by 50% (Muoio et al., 1999). Incubation of rat hepatocytes with AICAR resulted in a 29% to 43% decrease in mitochondrial and microsomal GPAT activity (Muoio et al., 1999). In addition, incubation of rat hepatocytes with purified recombinant AMPK decreased mitochondrial GPAT activity (Muoio et al., 1999). In rats, mitochondrial GPAT activity in liver and adipose decreased by 50% after exercise (Park et al., 2002a). However, exercise did not affect mitochondrial GPAT activity in muscle or microsomal GPAT activity in liver, adipose, or muscle.

Regulation of lipogenic transcription factors

Peroxisome proliferator-activated receptors are nuclear receptor proteins able to regulate the expression of lipogenic enzymes. The transcriptional co-activator p300 is able to mediate the activation of PPAR as well as other nuclear receptors (Gelman et al., 1999; Vo and Goodman, 2001). AMP-activated protein kinase can phosphorylate p300 on serine-89 causing a reduction in its affinity with various nuclear receptors (Yang et al., 2001). Yang and others (2001) transfected Baby Hamster Kidney cells with an expression vector producing PPARγ and a luciferase reporter plasmid containing a PPAR-response element. Incubated transfected cells with AICAR for 24 h resulted in a 75% decrease in the transcriptional activity of PPARγ.

Sterol regulatory element binding proteins are transcription factors able to regulate lipid homeostasis by controlling the expression of lipogenic enzymes including ACC and FAS. Activation of AMPK with AICAR or metformin significantly decreases insulin induced expression of SREBP1 mRNA in rat hepatocytes (Zhou et al., 2001). In addition, treatment of rat hepatocytes with these AMPK activators decreases the mRNA expression of FAS and Spot-14, two genes known to be regulated by SREBP1. Rat hepatoma H4IIEC3 cells treated with ethanol increase mature SREBP1 protein abundance by 2.2 fold (You et al., 2004). Addition of AICAR or metformin significantly blocked the induction of mature SREBP1 by ethanol. In support, incubating cultured pancreatic islets with AICAR decreased the expression of endogenous SREBP1 and FAS genes as well as reversed the effect of over-expressing mature SREBP1 on FAS mRNA expression and cellular TAG content (Diraison et al., 2004).

HYPOTHESIS STATMENT

In liver tissue, LXR and AMPK have the ability to regulate lipid synthesis by either modifying gene expression or by phosphorylating lipogenic enzymes. Our working hypothesis is that activation of LXR and AMPK will enhance or inhibit *de novo* fatty acid synthesis in bovine mammary epithelial cells, respectively. The identification of LXR and AMPK as regulators of *de novo* fatty acid synthesis will further enhance our understanding of milk fat synthesis in the mammary gland of the lactating dairy cow.

CHAPTER 3

Polyunsaturated Fatty Acids Inhibit *de novo* Fatty Acid Synthesis in Bovine Mammary Epithelial Cells

INTRODUCTION

In vitro, saturated, monounsaturated, and polyunsaturated fatty acids can regulate fatty acid synthesis in mammary epithelial cells. Treatment of bovine mammary epithelial cells with palmitic acid, a saturated fatty acid, stimulates the synthesis of butyric and palmitic acid (Hansen and Knudsen, 1987). In addition, oleic acid, a monounsaturated fatty acid, inhibited the synthesis and esterification of short- and medium-chain fatty acids. More recently, the effects of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-10, *cis*-12 CLA, polyunsaturated fatty acids, on fatty acid synthesis have been evaluated in MAC-T cells, a bovine mammary epithelial cell line (Peterson et al., 2004). Concurrent with *in vivo* data (Baumgard et al., 2000), Peterson and coworkers (2004) observed a significant decrease in *de novo* fatty acid synthesis and lipogenic gene expression in response to *trans*-10, *cis*-12 CLA. Similarly, incubation of MAC-T with increasing concentrations of oleic acid resulted in a decrease in acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) activity (Jayan and Herbein, 2000).

Sterol-regulatory element binding protein-1c (SREBP1c) is a membrane-bound transcription factor known to enhance fatty acid synthesis by activating the genes encoding ACC, FAS, glycerol-3-phosphate acyltransferase, and other lipogenic enzymes (Stoeckman and Towle, 2002). Exposure of mammary epithelial cells and rat hepatoma cells to unsaturated fatty acids decreases the expression of SREBP1c (Ou et al., 2001; Peterson et al., 2004). *In vivo,* the decreased expression of SREBP1 and the

coordinated reduction in SREBP1-responsive lipogenic enzymes is considered to play a central role in the regulation of milk fat synthesis in response to *trans*-10, *cis*-12 CLA (Harvatine and Bauman, 2006).

The use of an *in vitro* model can be used to investigate the molecular mechanisms responsible for milk fat depression *in vivo*. To date, MAC-T cells have been the most heavily investigated bovine mammary epithelial cell line. However, in addition to MAC-T, another bovine mammary epithelial cell line (BME-UV) has been characterized (Zavizion et al., 1996). Whether polyunsaturated fatty acids can regulate fatty acid synthesis has yet to be evaluated in BME-UV cells. Therefore, our objective was to evaluate the effect of polyunsaturated fatty acids on *de novo* fatty acid synthesis, mRNA abundance for lipogenic enzymes, and the potential involvement of SREBP1 in the BME-UV bovine mammary epithelial cell line.

MATERIALS AND METHODS

Cell culture and treatments

Experiments utilized BME-UV cells (Zavizion et al., 1996). BME-UV bovine mammary epithelial cells were compared to MAC-T cells (Huynh et al., 1991) when evaluating the effects of polyunsaturated fatty acids on *de novo* fatty acid synthesis. Throughout the duration of the experiments, cells were cultured at 37°C in 5% CO_2, and the medium was changed every 24 h. Cells were seeded on gelatin coated cell culture plates at a density of 5×10^5 cells/cm^2 and 2.6×10^4 cells/cm^2 for BME-UV and MAC-T cells, respectively. Cells were grown in basal medium (Dulbecco's Modified Eagle's Medium with 10 kU/ml penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B)

supplemented with 10% fetal bovine serum. Once cells reached confluency (approximately 72 h post seeding) serum was removed and hormones (0.1 µg/mL insulin and 1.5 µg/mL prolactin; Sigma Chemical Co., St. Louis, MO) were added to the basal media. Cells were cultured in basal media with hormones for 24 h and then treatments were applied.

In all experiments, cells were treated with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (Nu-Chek Prep, Inc., Elysian, MN, Matreya, LLC, Pleasant Gap, PA) for 24 h. All fatty acids were complexed with fatty acid free bovine serum albumin (BSA; Sigma Chemicals Co., St. Louis, MO; 1:3 molar ratio of BSA:fatty acid). Fatty acids were bound to BSA according to Ip and coworkers (1999) with the following modifications. Pure fatty acid was incubated with preheated sodium hydroxide (70°C) in a 1:1 molar ratio and vortexed periodically, until clear. Sodium salts were diluted in warm (37°C) basal medium with hormones in a 3:1 molar ratio with BSA. Complexes in media were filtered using a 0.22 µm syringe filter prior to utilization. Bovine serum albumin served as control when evaluating the effects of fatty acids.

Fatty acid synthesis assay

De novo fatty acid synthesis in BME-UV or MAC-T was determined by quantifying the incorporation of [1-^{14}C]-labeled acetate into lipid. Methods were adapted from Peterson and coworkers (2004) and modified as described. Cells were cultured in 6-well cell culture plates and treated with BSA or fatty acids. After 24 h of treatment, cells were incubated with ^{14}C-labeled acetate (MP Biomedicals, Solon, OH) for a period of 4 h. During this 4 h period, cells were cultured in 3 mM acetate (0.8 µCi/µmol).

Following the 4 h incubation with [1-^{14}C]-labeled acetate, cells were lysed in wells with sodium dodecyl sulfate buffer (0.1% in phosphate buffered saline). Lipid within the lysate was then extracted using hexane:isopropanol (3:2). The solvent layer was combined with 16 ml of scintillation cocktail (Scintisafe 30% Cocktail; Fisher Scientific, Pittsburgh, PA) for quantification of label incorporation into lipid using a LS 6000LL Beckmann scintillation counter. Activity was calculated and expressed as pmol of acetate incorporated per µg of DNA.

Real time PCR

Cultured BME-UV cells were lysed in 6-well culture plates using 1 mL of TRI Reagent (Molecular Research Center Inc.; Cincinnati, OH) per well. Total RNA was isolated according to manufacturer's instructions. Ribonucleic acid pellets were resuspended in RNase-free water, and quantified at 260 nm using a spectrophotometer. Total RNA was reverse transcribed (500 ng per reaction) into cDNA using the Omniscript reverse transcription kit (Qiagen; Valencia, CA) according to manufacturer's instructions using oligo(dT) (Roche Applied Science; Indianapolis, IN) as the primer.

Real-time PCR reactions were performed using the Quantitect SYBR Green PCR kit (Qiagen; Valencia, CA) and an Applied Biosystems 7300 Real-time PCR machine (Applied Biosystems; Foster City, CA). Quantification of gene transcripts for ACCα, FAS, peroxisome-proliferator activated receptor-γ (PPARγ), liver X receptor-α (LXRα), and SREBP1 was completed using gene-specific primers (Table 3.1). β-actin was used as the endogenous control gene. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Reaction conditions were as follows: 1 cycle at 95°C for

Table 3.1. Primer sequences used to detect ACCα, FAS, PPARγ, LXRα, SREBP1, and β-actin mRNA[1].

Target Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')	Product Size (bp)
ACCα	GGGTGAAAGACTGGGTTGGAA	GTAGTGTAGGCACGAGACAG	172
FAS	AATGACCACTTTGCCGATGT	TGAAGGACGGTGTACCACAA	152
PPARγ	CATCTTCCAGGGGTGTCAGT	TCCTACCCCAGGAGTATAGG	186
LXRα	TCAACCCCATCTTCGAGTTC	ACGACTACTTTGACCACTCG	232
SREBP1[2]	ATGCCATCGAGAAACGCTAC	CTCTTGGACTCAGACGCCTG	180
β-actin	CTCTTCCAGCCTTCCTTCCT	CGTCTTTCTCTAGTGACGGG	178

[1]ACCα, acetyl-CoA carboxylase-α; FAS, fatty acid synthase; PPARγ, peroxisome proliferator-activated receptor-γ; LXRα, liver X receptor-α; and SREBP1, sterol regulatory element binding protein-1.
[2]Primer pairs do not distinguish between SREBP1a and SREBP1c isoforms.

10 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Each reaction was performed in duplicate wells.

DNA quantification

Bovine mammary epithelial cells (BME-UV or MAC-T) were harvested in DNA assay buffer (Tris Base, EDTA, NaCl; pH 7.4) and then sonicated (2 × 5 s) on ice. Deoxyribonucleic acid concentration of cell lysates was assayed as described by Labarca and Paigen (1980) with the following modifications. Briefly, 2 µL of cell lysate was transferred to a cuvette followed by 1998 µL of DNA assay buffer to yield a total assay volume of 2 mL. Samples were measured in triplicate using a DyNA Quant 200 fluoremeter (Hoefer Pharmacia Biotech; San Francisco, CA). Calf thymus DNA (Sigma Chemical Co.; St. Louis, MO) was used as the standard.

Cellular fractionation

For determination of ACCα and premature SREBP1 (pSREBP1) protein abundance, BME-UV cells were seeded on 6-well cell culture plates. Following a wash with phosphate buffered saline, cells were harvested in ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA, and protease inhibitor cocktail) for 15 min on ice. Lysate was centrifuged at 14,000 × *g* at 4°C for 10 min. The resulting supernatant was used for ACCα and pSREBP1 protein detection.

For determination of mature (mSREBP1) protein, BME-UV cells were seeded on 100-mm cell culture dishes. Cells were harvested and processed as previously described by DeBose-Boyd and coworkers (1999) with the following modifications.

Briefly, cells were harvested in media from two dishes, and centrifuged at 250 × *g* for 5 min at 4°C. The supernatant was removed and cells were washed with ice-cold phosphate buffered saline and centrifuged at 250 × *g* for 5 min at 4°C. The cell pellet was resuspended in 0.6 ml of buffer A (10 mM HEPES-KOH at pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 250 mM sucrose, and protease inhibitor cocktail), passed through a 25-gauge needle 25 times, and centrifuged at 1,000 × *g* for 5 min at 4°C. The resulting pellet was resuspended in 0.1 ml of buffer B (20 mM HEPES-KOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, and protease inhibitor cocktail), rocked at 4°C for 1 hr, and centrifuged at 100,000 × *g* for 15 min at 4°C. The supernatant from this centrifugation was designated the nuclear extract.

Immunoblotting

Cell fractionation supernatants were assayed for protein concentration using the Bradford assay (Bio-Rad; Hercules, CA). To ensure equal loading, samples were diluted to the same protein concentrations with Laemmli sample buffer (Bio-Rad; Hercules, CA) and heated at 95°C for 7 min. Acetyl-CoA carboxylase-α and SREBP1 were separated by electrophoresis using 7.5% or 12% polyarcylamide gels (Cambrex Corporation, East Rutherford, NJ), respectively. Proteins were transferred to a PVDF membrane using a Bio-Rad trans-blot SD semi-dry transfer cell (Bio-Rad; Hercules, CA). Membranes were blocked in blocking buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween and 5% dried nonfat milk) on a rocker. In blocking buffer, primary anti-ACC antibody (Cell Signaling Technology; Beverly, MA) or anti-SREBP1 antibody

(Santa Cruz Biotechnology, Santa Cruz, CA) were applied at 1:1000 or 1:750, respectively. Membranes were then washed in blocking buffer. Following wash, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2000) or anti-mouse antibodies (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for ACC and SREBP1 detection, respectively. Proteins were detected using ECL-Plus chemiluminescence substrate (Amersham Biosciences; Pittsburg, PA) according to manufacturer's instructions. Chemiluminescence was measured using a Chemidoc XRS digital imaging system and densitometry was quantified using Quantity One software (Bio-Rad; Hercules, CA).

Statistical analysis

Data are reported as least squares means ± SEM unless otherwise noted. All data were analyzed using the Mixed procedure of SAS (SAS for Windows Version 9.1, SAS Institute Inc., Cary, NC). When analyzing the abundance of mSREBP1 in BME-UV, the model included the fixed effects of treatment and set. For analysis of all other data, the model included the fixed effects of treatment, set, and treatment by set interaction. Replicate within set was the random effect. Treatments included the presence the presence of fatty acids. One set represents one independent experiment. If a significant treatment effect was observed, Tukey's multiple comparison procedure was used to separate treatment means. Real-time PCR data were analyzed using original ΔCt values normalized with β-actin as the endogenous control gene. For the purpose of presentation, least squares means are illustrated as fold change ($2^{-\Delta\Delta Ct}$) relative to control.

RESULTS

In the bovine mammary gland, fatty acids can regulate milk fat synthesis by controlling the transcription of lipogenic genes. It has been well documented that the inhibition of milk fat synthesis in the lactating dairy cow is caused by various isomers of CLA including *trans*-10, *cis*-12. In support of *in vivo* data, we observed significant reductions in the incorporation of acetate into lipid in response to *trans*-10, *cis*-12 CLA treatment in MAC-T (Figure 3.1); however, we observed similar reductions in response to LA and *cis*-9, *trans*-11CLA (Figure 3.1). We examined another bovine mammary epithelial cell line for its response to polyunsaturated fatty acids. Incubation of BME-UV cells with 50 µM LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA significantly reduced the incorporation of acetate into fatty acids (Figure 3.2).

We next focused on the mechanism of polyunsaturated fatty acid regulation of fatty acid synthesis. Acetyl-CoA carboxylase-α and FAS are two lipogenic enzymes required for *de novo* fatty acid synthesis in the bovine mammary epithelial cell. The mRNA expression of ACCα was unaffected by LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (Figure 3.3); however, polyunsaturated fatty acid treatment significantly decreased ACCα protein abundance (Figure 3.4). Treatment of BME-UV with LA or *trans*-10, *cis*-12 CLA decreased the mRNA expression of FAS (Figure 3.3); however, the mRNA expression of FAS was unaffected by *cis*-9, *trans*-11 CLA (Figure 3.3).

In the bovine mammary gland, SREBP1 is considered the predominant transcriptional regulator of lipid synthesis. Sterol regulatory element binding protein-1 regulates the transcription of various lipogenic enzymes including ACCα and FAS. In the present study, treatment of BME-UV with LA or *trans*-10, *cis*-12 CLA significantly

Figure 3.1. Effect of treating bovine mammary epithelial cells (MAC-T) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), *trans*-10, *cis*-12 CLA (10,12 CLA), or in the absence of fatty acid (No FA; control) for 24 h on *de novo* fatty acid synthesis. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences are signified by differing superscripts, $P < 0.0001$.

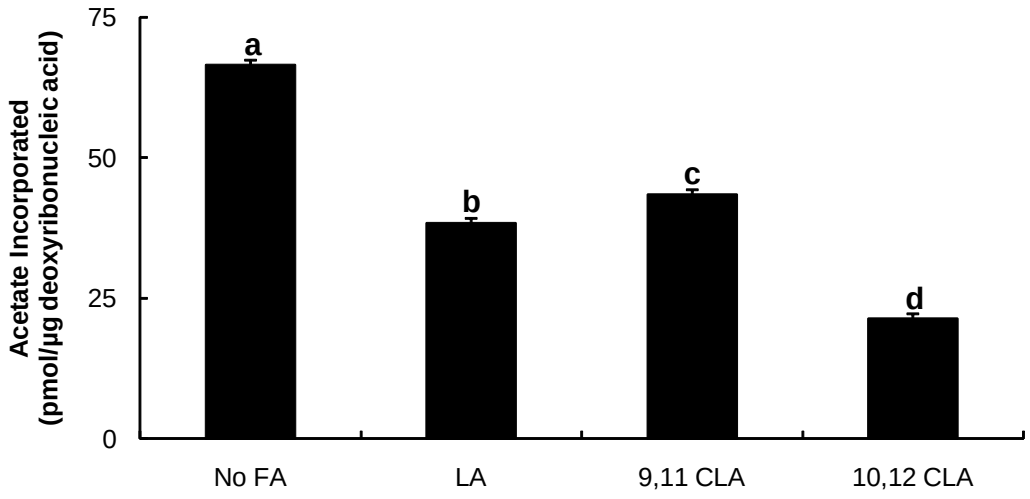


Figure 3.2. Effect of treating bovine mammary epithelial cells (BME-UV) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), or *trans*-10, *cis*-12 CLA (10,12 CLA), or in the absence of fatty acid (No FA; control) for 24 h on *de novo* fatty acid synthesis. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences are signified by differing superscripts, $P < 0.0001$.

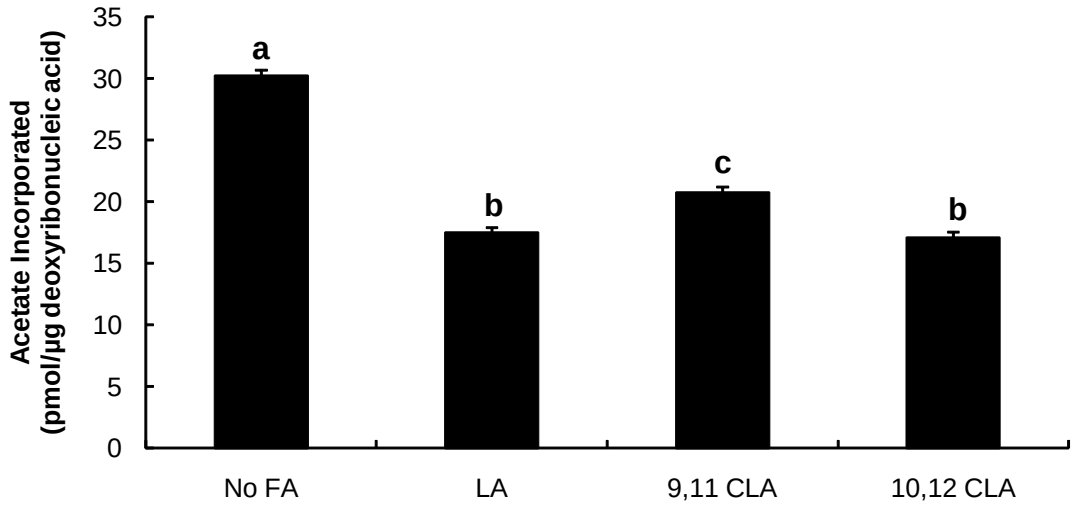


Figure 3.3. Effect of treating bovine mammary epithelial cells (BME-UV) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), *trans*-10, *cis*-12 CLA (10,12 CLA), or in the absence of fatty acid (No FA; control) for 24 h on lipogenic gene expression. Lipogenic genes evaluated were (A) acetyl-CoA carboxylase-α (ACCα) and (B) fatty acid synthase (FAS). Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. Differences in ΔCt values for treatments are signified by differing superscripts within transcript, $P < 0.05$.

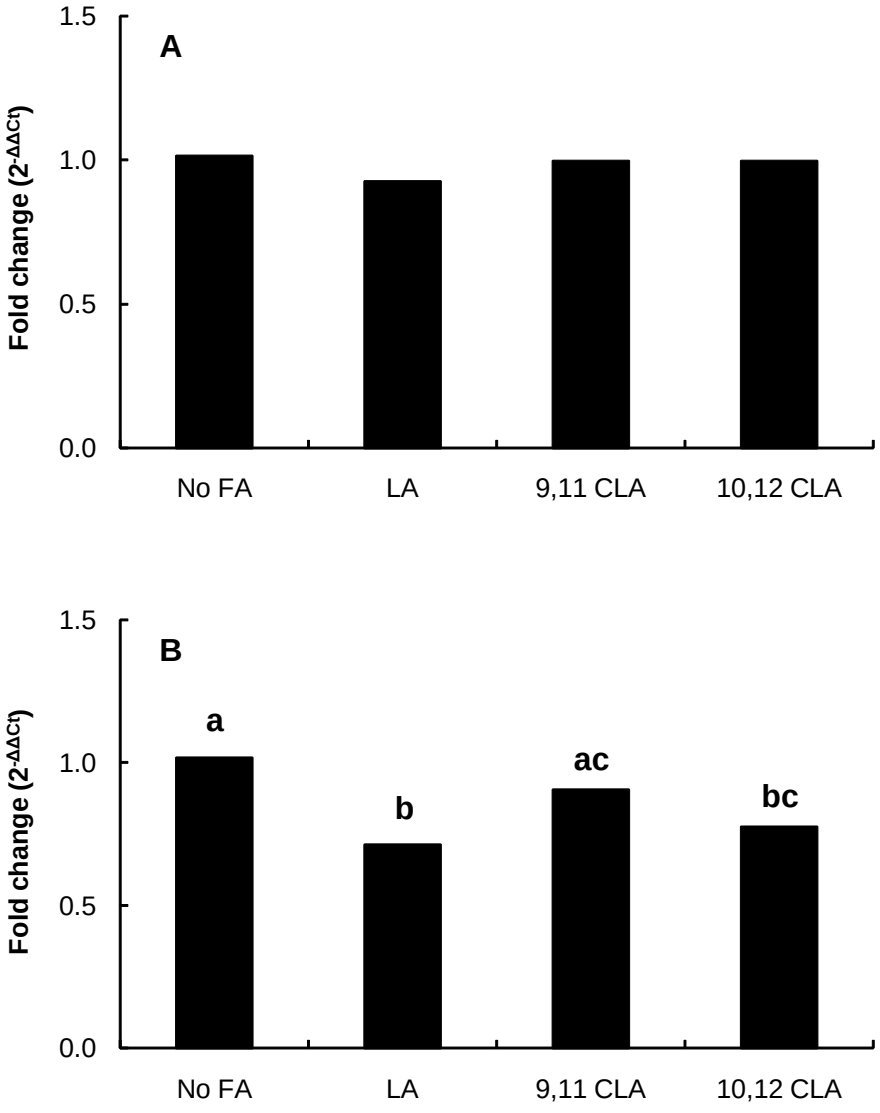
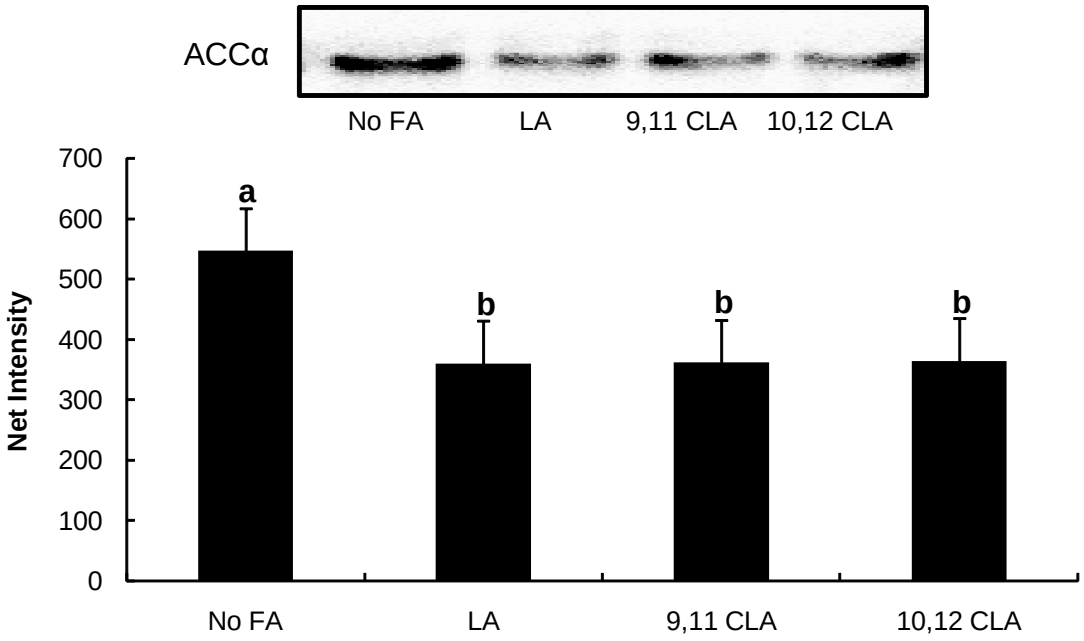


Figure 3.4. Effect of treating bovine mammary epithelial cells (BME-UV) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), *trans*-10, *cis*-12 CLA, or in the absence of fatty acid (No FA; control) for 24 h on acetyl-CoA carboxylase-α (ACCα) protein abundance. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences within transcript are signified by differing superscripts, $P < 0.10$.



decreased the mRNA expression of SREBP1 (Figure 3.5); however, the mRNA expression of SREBP1 was unaffected by *cis*-9, *trans*-11 CLA (Figure 3.5). In addition, treatment of BME-UV with LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA resulted in the reduction of pSREBP1 and mSREBP1 protein abundance (Figure 3.6). Peroxisome proliferator-activated receptor-γ and LXRα are two other lipogenic transcription factors expressed in the mammary gland. The mRNA expression of PPARγ was unaffected by polyunsaturated fatty acid treatment (Figure 3.5). The mRNA expression of LXRα increased in response to treating BME-UV with *trans*-10, *cis*-12 CLA (Figure 3.5). However, treatment of BME-UV with either LA or *cis*-9, *trans*-11 CLA failed to affect the mRNA expression of LXRα (Figure 3.5).

DISCUSSION

The inhibitory effects of *trans*-10, *cis*-12 CLA on milk fat production and lipogenic gene expression in the bovine mammary gland have been well established (Baumgard et al., 2002; Harvatine and Bauman, 2006). Abomasal infusions of *trans*-10, *cis*-12 CLA have resulted in ~40-50% reductions in lipogenic enzyme mRNA expression including ACC, FAS, glycerol-3-phosphate acyltransferase, and lipoprotein lipase (Baumgard et al., 2002). Sterol regulatory element binding protein-1 is a known regulator of these enzymes (Lopez et al., 1996; Magana and Osborne, 1996); therefore, it has been proposed that SREBP1 may be responsible for regulating their expression in response to *trans*-10, *cis*-12 CLA (Peterson et al., 2004; Harvatine et al., 2009). In support of this hypothesis, the mRNA expression of SREBP1 is significantly decreased in response to *trans*-10, *cis*-12 CLA treatment in lactating cows (Harvatine and Bauman, 2006).

Figure 3.5. Effect of treating bovine mammary epithelial cells (BME-UV) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), *trans*-10, *cis*-12 CLA (10,12 CLA), or in the absence of fatty acid (No FA; control) for 24 h on lipogenic transcription factor gene expression. Genes involved in the transcriptional regulation of lipogenic enzymes included (A) peroxisome proliferator-activated receptor-γ (PPARγ), (B) liver X receptor-α (LXRα), and (C) sterol regulatory element binding protein-1 (SREBP1). Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. Differences in ΔCt values for treatments are signified by differing superscripts within transcript, $P < 0.01$.

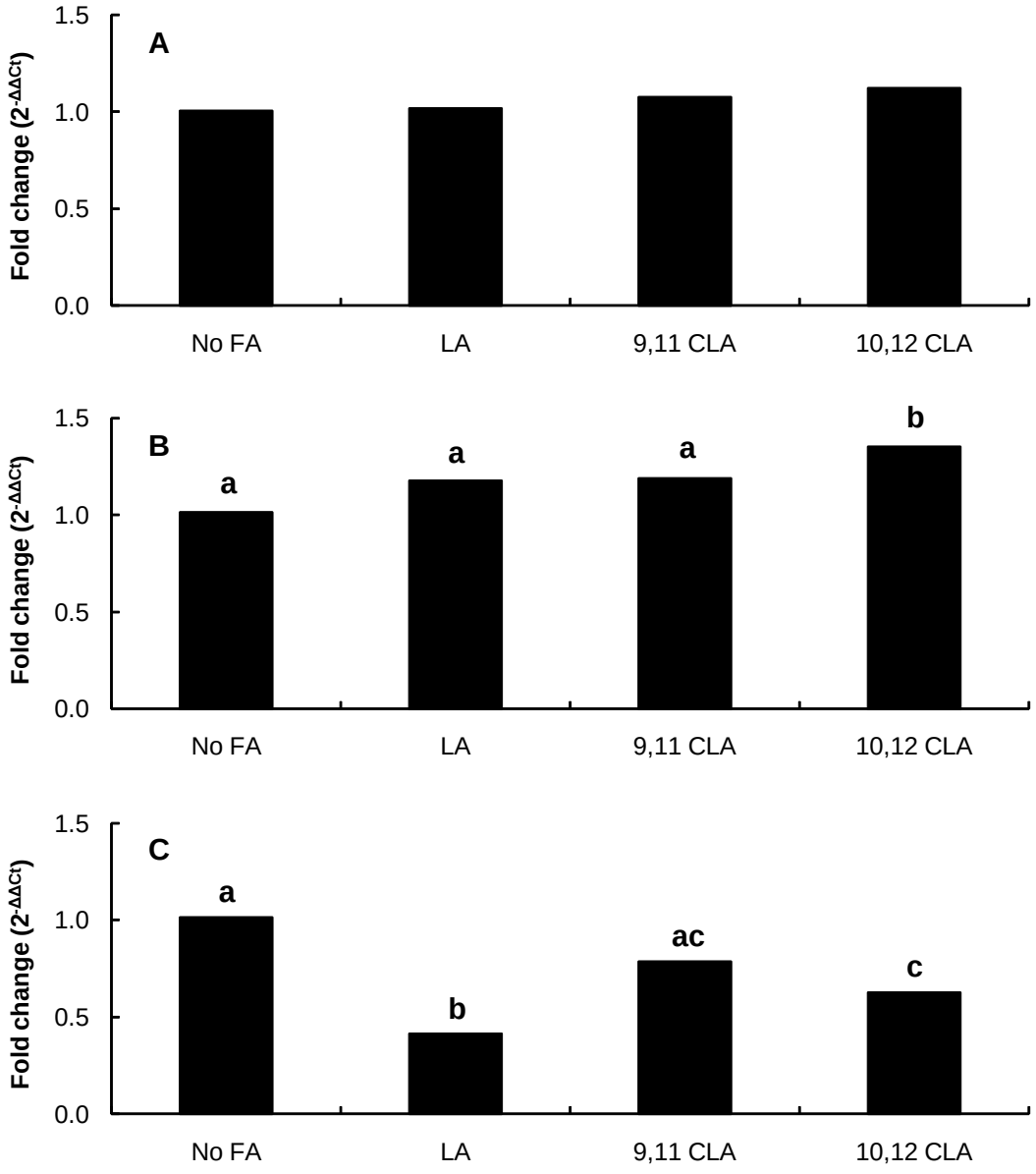
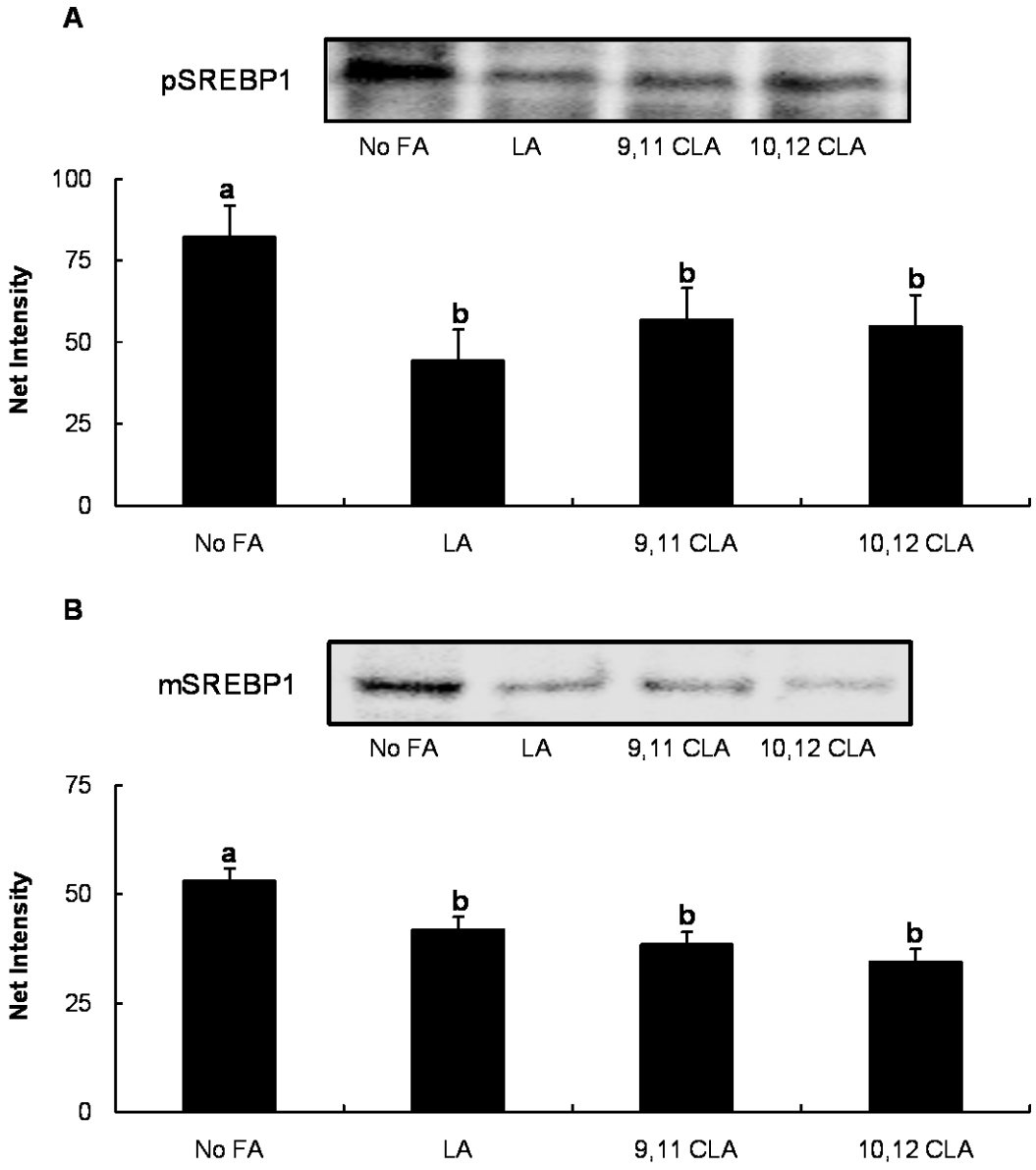


Figure 3.6. Effect of treating bovine mammary epithelial cells (BME-UV) with 50 µM linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), *trans*-10, *cis*-12 CLA, or in the absence of fatty acid (No FA; control) for 24 h on premature sterol regulatory element binding protein-1 (pSREBP1) and mature sterol regulatory element binding protein-1 (mSREBP1) protein abundance. (A) Effects of treatment on pSREBP1 protein abundance. (B) Effects of treatment on mSREBP1 protein abundance. For pSREBP1, error bars represent SEM for two independent experiments with three replicates per experiment. For mSREBP1, error bars represent SEM for two independent experiments. Treatment differences within either pSREBP1 or mSREBP1 are signified by differing superscripts, $P < 0.05$.



In dairy cows, the ability of specific CLA isomers to inhibit milk fat synthesis seems to be unique to these polyunsaturated fatty acids. Saturated fatty acids have been shown to have no effect on milk fat production (Drackley et al., 1992; Bremmer et al., 1998). Oleic acid, a monounsaturated fatty acid, fails to inhibit milk fat synthesis (Gaynor et al., 1994; Lacount et al., 1994). In addition, LA alone fails to inhibit milk fat production in the absence of CLAs (Loor and Herbein, 1998). In the present study, we established that 50 µM LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA reduced *de novo* fatty acid synthesis in the BME-UV and MAC-T bovine mammary epithelial cell lines. We also observed similar decreases in *de novo* fatty acid synthesis in response to stearic acid, a saturated fatty acid, and oleic acid (data not shown). In support, Hansen and Knudsen (1987) observed, *in vitro*, that the effect of oleic acid addition on the synthesis and esterification of short- and medium-chain fatty acids was strongly inhibitory. Recently, treatment of MAC-T with 75 µM *trans*-10, *cis*-12 CLA resulted in a 50% reduction in the incorporation of radiolabeled acetate (Peterson et al., 2004).

Incubating BME-UV with polyunsaturated fatty acids inhibits *de novo* fatty acid synthesis by modifying lipogenic gene expression. In response to the inhibitory effect of polyunsaturated fatty acids in BME-UV, we observed a reduction in FAS mRNA expression and ACCα protein abundance. Abomasal infusions of *trans*-10, *cis*-12 CLA have resulted in ~40-50% reductions in ACC and FAS mRNA expression (Baumgard et al., 2002). Liver X receptor-α, PPARγ, and SREBP1 are known regulators of lipogenic gene expression in the mammary gland. In BME-UV, treatment with polyunsaturated fatty acids did not modify the expression of LXRα. In addition, the mRNA expression of PPARγ was not modified by LA or *cis*-9, *trans*-11 CLA; however, treatment with *trans*-

10, *cis*-12 CLA increased PPARγ mRNA expression. Harvatine and Bauman (2007) observed that the expression of LXRα and PPARγ was not modified by intravenous infusion of *trans*-10, *cis*-12 CLA. Whether or not treatment with *trans*-10, *cis*-12 CLA modifies the activity of these nuclear receptors has yet to be determined. Contrary to LXRα and PPARγ, SREBP1 is considered to be the primary regulator of milk fat synthesis in response to *trans*-10, *cis*-12 CLA (Peterson et al., 2004; Harvatine et al., 2009). Incubation of BME-UV with 50 µM LA or *trans*-10, *cis*-12 CLA decreased SREBP1 mRNA expression in addition to the abundance of pSREBP1 and mSREBP1. Treatment of BME-UV cells with 50 µM cis-9, trans-11 CLA was also able to inhibit the abundance of pSREBP1 and mSREBP1. Harvatine and Bauman (2006) also observed a decrease in SREBP1 mRNA expression in response to *trans*-10, *cis*-12 CLA treatment in lactating dairy cows. Contrary to Harvatine and Bauman (2006), Peterson and coworkers (2004) observed no significant difference in the mRNA expression of SREBP1 between *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA treatments compared to a BSA control. When comparing *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, *trans*-10, *cis*-12 CLA increased pSREBP1 protein and decreased mSREBP1 compared to *cis*-9, *trans*-11 CLA (Peterson et al., 2004).

Understanding the mechanisms responsible for the differences observed *in vitro* compared to *in vivo* data is difficult. Keating and coworkers (2008) treated MAC-T with increasing concentrations of either *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. They observed a reduction in cell number and a 2-fold increase in induction of apoptosis at concentrations of 35 µM and above. To rule out cell death as a plausible explanation for our observed decrease in *de novo* fatty acid synthesis in response to fatty acids, we

measured cell viability using trypan blue dye exclusion. Culture viability was estimated at approximately 97% in response to treating BME-UV with stearic acid, oleic acid, LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (data not shown). Bauman and Davis (1974) discussed the possibility that the suppression of *de novo* fatty acid synthesis observed *in vitro* is due to the detergent properties of long-chain fatty acids. However, Wright and coworkers (2002) concluded that the inhibitory effect of palmitic acid on fatty acid synthesis in bovine mammary tissue was not the result of a detergent effect. Finally, Jayan and Herbein (2000) propose that long-chain unsaturated fatty acids may influence cell membrane fluidity. Fatty acids provided in the media of cultured mammary epithelial cells are primarily incorporated into cell membrane phospholipids (Baughman, 1995). The amount of saturated and unsaturated fatty acids, as well as the chain length of fatty acids, determines membrane fluidity. Short- and medium-chain saturated fatty acids and long-chain unsaturated fatty acids enhance cell membrane fluidity. Jayan and Herbein (2000) suggest that the presence of long-chain unsaturated fatty acids inhibits the synthesis of short- and medium-chain saturated fatty acids in order to maintain membrane fluidity. In the present study, it is possible that the presence of polyunsaturated fatty acids inhibited *de novo* fatty acid synthesis in order to maintain membrane fluidity.

In the present study, we demonstrated that the BME-UV bovine mammary epithelial cell line expresses SREBP1 and that reductions in *de novo* fatty acid synthesis by polyunsaturated fatty acids results in the downregulation of SREBP1 and SREBP1 target genes. Preliminary evidence also suggests no difference between the BME-UV and MAC-T bovine mammary epithelial cell lines in response to fatty acids varying in

degree of saturation. Further research is needed to better understand the molecular mechanisms responsible for controlling fatty acid synthesis *in vitro* and *in vivo*.

CHAPTER 4

Activation of Liver X Receptor Enhances *de novo* Fatty Acid Synthesis in Bovine Mammary Epithelial Cells

INTRODUCTION

Liver X receptors (LXR) are nuclear receptors that can regulate the synthesis of lipid and control cholesterol homeostasis upon activation by oxysterols or synthetic agonists (Lehmann et al., 1997; Schultz et al., 2000). Liver X receptor-α and LXRβ are two known isoforms (Song et al., 1994; Willy et al., 1995) and both regulate the transcription of lipogenic enzymes by binding to DNA in a heterodimeric complex with retinoid X receptor (RXR; Willy et al., 1995). Mice carrying a mutated LXRα gene have decreased expression of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and sterol regulatory element binding protein-1 (SREBP1), cellular proteins with significant roles in the synthesis of fatty acids (Peet et al., 1998b).

Sterol regulatory element binding protein-1 is a membrane-bound transcription factor that directly regulates the synthesis and uptake of cholesterol and fatty acids (see review by Brown and Goldstein (1997)). The promoters of the lipogenic enzymes ACC, FAS, and glycerol-3-phosphate acyltransferase (GPAT) all contain a sterol response element (SRE) capable of binding SREBP1 (Lopez et al., 1996; Magana and Osborne, 1996; Ericsson et al., 1997). Interestingly, the promoter of SREBP1c contains a liver X response element (LXRE) for LXRα and LXRβ suggesting the potential for transcriptional regulation of SREBP1c by LXRs (Yoshikawa et al., 2001).

Sterol regulatory element binding protein-1 is responsive to LXR activation. In rat hepatoma cells, transcription of SREBP1c was stimulated by oxysterols that activate LXRα and LXRβ (DeBose-Boyd et al., 2001). Incubation of human preadipocytes with

T0901317 (T09), a LXR agonist, increased ACC, FAS, and SREBP1c mRNA expression (Darimont et al., 2006). In addition, activation of LXR by T09 resulted in increased relative amounts of hepatic SREBP1, ACC, FAS, and GPAT mRNA and this effect was markedly reduced in SREBP1c knockout mice indicating an essential role of SREBP1c in the LXR response (Liang et al., 2002). In addition to LXR indirectly promoting the transcription of ACC and FAS by increasing the expression of SREBP1c, LXR can regulate the transcription of ACC and FAS by binding to a LXRE found within the promoter region of these lipogenic genes (Joseph et al., 2002; Talukdar and Hillgartner, 2006). In SREPB1c knockout mice, Liang and coworkers (2002) still observed an increase in ACC and FAS mRNA expression in response to T09 suggesting a direct effect on ACC and FAS mRNA expression by LXR.

Sterol regulatory element binding protein-1 is one of the primary regulators of mammary lipid synthesis during diet-induced milk fat depression and treatment with conjugated linoleic acid (CLA; Harvatine and Bauman, 2006); however, the role of LXR in mammary lipid synthesis is unknown. In dairy cows, LXRα, but not LXRβ expression is increased during lactation compared to nonlactating mammary tissue (Harvatine and Bauman, 2007). In addition, Farke and coworkers (2008) have identified the presence of LXRα in bovine mammary tissue, but did not elucidate its role in lipid metabolism. Therefore, our objective was to evaluate the effect of LXR activation on *de novo* fatty acid synthesis in bovine mammary epithelial cells.

MATERIALS AND METHODS

Cell culture and treatments

In vitro experiments utilized BME-UV bovine mammary epithelial cells (Zavizion et al., 1996). Throughout the duration of the experiments, cells were cultured at 37°C in 5% CO_2, and the medium was changed every 24 h. All cells were seeded on plastic cell culture plates at a density of 5×10^5 cells/cm^2. Cells were grown in basal medium (Dulbecco's Modified Eagle's Medium with 10 kU/ml penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B) supplemented with 10% fetal bovine serum. Once cells reached confluency (approximately 72 h post seeding), serum was removed and hormones (0.1 µg/mL insulin and 1.5 µg/mL prolactin; Sigma Chemical Co., St. Louis, MO) were added to the basal media. Cells were cultured in basal media with hormones for 24 h and then treatments were applied.

Cells were treated with T09 (2 µM; Sigma Chemical Co., St. Louis, MO) for 8 (acute) or 24 h (chronic). In a separate experiment, cells were treated with T09 (2 µM) with or without 10 µM geranylgeranyl pyrophosphate (GGPP; Sigma Chemical Co., St. Louis, MO) for 24 h. Dimethyl sulfoxide (DMSO; Sigma Chemicals Co., St. Louis, MO) served as control when evaluating the effects of T09 or GGPP. Cells were treated with no more than 0.4% DMSO.

When evaluating the effects of fatty acids in combination with T09 on *de novo* fatty acid synthesis, cells were treated with fatty acids and T09 for 24 and 8 h, respectively. Fatty acids included linoleic acid (LA), *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA (Nu-Chek Prep, Inc., Elysian, MN, Matreya, LLC, Pleasant Gap, PA). All fatty acids were complexed with fatty acid free bovine serum albumin (BSA; Sigma

Chemicals Co., St. Louis, MO; 1:3 molar ratio of BSA:fatty acid). Fatty acids were bound to BSA according to Ip and coworkers (1999) with the following modifications. Pure fatty acid was incubated with preheated sodium hydroxide (70°C) in a 1:1 molar ratio and vortexed periodically, until clear. Sodium salts were diluted in warm (37°C) basal medium with hormones in a 3:1 molar ratio with BSA. Complexes in media were filtered using a 0.22 µm syringe filter prior to utilization. Bovine serum albumin served as control when evaluating the effects of fatty acids.

Fatty acid synthesis assay

De novo fatty acid synthesis was determined by quantifying the incorporation of [1-^{14}C]-labeled acetate into lipid. Methods were adapted from Peterson and coworkers (2004) and modified as described. After 8 or 24 h of treatment, cells cultured in 12-well culture plates, were incubated with ^{14}C-labeled acetate (MP Biomedicals, Solon, OH) for a period of 4 h. During this 4 h period, cells were cultured in 3 mM acetate (0.37 µCi/µmol). Following 4 h incubation with [1-^{14}C]-labeled acetate, cells were lysed in wells with sodium dodecyl sulfate buffer (0.1% in phosphate buffered saline). Lipid within the lysate was then extracted using hexane:isopropanol (3:2). The solvent layer was combined with 16 mL of scintillation cocktail (Scintisafe 30% Cocktail; Fisher Scientific, Pittsburgh, PA) for quantification of label incorporation into lipid using a LS 6000LL Beckmann scintillation counter. Activity was calculated and expressed as pmol of acetate incorporated per µg of DNA.

Real time PCR

Following either acute or chronic treatment with T09, cells were lysed in 6-well culture plates using 1 mL of TRI Reagent (Molecular Research Center Inc.; Cincinnati, OH) per well. Total RNA was isolated according to manufacturer's instructions. RNA pellets were resuspended in RNase-free water, and quantified at 260 nm using a spectrophotometer. Total RNA was reverse transcribed (500 ng per reaction) into cDNA using the Omniscript reverse transcription kit (Qiagen; Valencia, CA) according to manufacturer's instructions using oligo(dT) (Roche Applied Science; Indianapolis, IN) as the primer.

Real-time PCR reactions were performed using the Quantitect SYBR Green PCR kit (Qiagen; Valencia, CA) and an Applied Biosystems 7300 Real-time PCR machine (Applied Biosystems; Foster City, CA). Quantification of gene transcripts FAS, LXRα, SREBP1, insulin-induced gene-1 (INSIG1), insulin-induced gene-2 (INSIG2), ATP-binding cassette transporter-G1 (ABCG1), and cytochrome P4501A1 (Cyp1A1) was completed using gene-specific primers (Table 4.1). β-actin was used as the endogenous control gene. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Reaction conditions were as follows: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Each reaction was performed in duplicate wells.

DNA quantification

Cells were harvested in DNA assay buffer (Tris Base, EDTA, NaCl; pH 7.4) then sonicated (2 × 5 s) on ice. Deoxyribonucleic acid concentration of cell lysates was

Table 4.1. Primer sequences used to detect FAS, LXRα, SREBP1, INSIG1, INSIG2, ABCG1, Cyp1A1, and β-actin mRNA[1].

Target Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')	Product Size (bp)
FAS	AATGACCACTTTGCCGATGT	TGAAGGACGGTGTACCACAA	152
LXRα	TCAACCCCATCTTCGAGTTC	ACGACTACTTTGACCACTCG	232
SREBP1[2]	ATGCCATCGAGAAACGCTAC	CTCTTGGACTCAGACGCCTG	180
INSIG1	GTCATCGCCACCATCTTCTC	AGTGGAACCTCTCGGTGTGTT	115
INSIG2	TCCAGTGTGATGCGGTGTGTA	AGTGTGACCGACGTGATAGTT	108
ABCG1	GACTCGGTCCTCACGCAC	CGGAGAAACACGCTCATCTC	231
Cyp1A1	CCGACCTCTACAGCTTCACC	CTTGGCCTCCTTGTTCACAT	185
β-actin	CTCTTCCAGCCTTCCTTCCT	CGTCTTTCTCTAGTGACGGG	178

[1]FAS, fatty acid synthase; LXRα, liver X receptor-α; SREBP1, sterol regulatory element binding protein-1; INSIG1, insulin induced gene-1; INSIG2, insulin-induced gene-2; ABCG1, ATP-binding cassette transporter-G1; and Cyp1A1, cytochrome P4501A1.
[2]Primer pairs do not distinguish between SREBP1a and SREBP1c isoforms.

assayed as described by Labarca and Paigen (1980) with the following modifications. Briefly, 2 µL of cell lysate were placed into a cuvette followed by 1998 µL of DNA assay buffer to yield a total assay volume of 2 mL. Samples were measured in triplicate using a DyNA Quant 200 fluoremeter (Hoefer Pharmacia Biotech; San Francisco, CA). Calf thymus DNA (Sigma Chemical Co.; St. Louis, MO) was used as a standard.

Cellular fractionation

For determination of premature and mature SREBP1 (pSREBP1 and mSREBP1, respectively) proteins, BME-UV cells were seeded on 100 mm cell culture dishes. Cells were harvested and processed as previously described by DeBose-Boyd and coworkers (1999) with the following modifications. Briefly, cells were harvested in media from two dishes, and centrifuged at 250 × *g* for 5 min at 4°C. The supernatant was removed and cells were washed with ice-cold phosphate buffered saline and centrifuged at 250 × *g* for 5 min at 4°C. The cell pellet was resuspended in 0.6 ml of buffer A (10 mM HEPES-KOH at pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 250 mM sucrose, and protease inhibitor cocktail), passed through a 25-gauge needle 25 times, and centrifuged at 1,000 × *g* for 5 min at 4°C. The resulting pellet was resuspended in 0.1 ml of buffer B (20 mM HEPES-KOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, and protease inhibitor cocktail), rocked at 4°C for 1 hr, and centrifuged at 100,000 × *g* for 15 min at 4°C. The supernatant from this centrifugation was designated the nuclear extract. The supernatant from the original 1,000 × *g* spin was used to prepare the membrane fraction by centrifugation at 10,000 × *g* for 15 min at 4°C. The resulting membrane pellets were resuspended in 0.1 ml of ice-cold lysis buffer

(50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA, and protease inhibitor cocktail).

Immunoblotting

Cell fractionation supernatants were assayed for protein concentration using the Bradford assay (Bio-Rad; Hercules, CA). To ensure equal loading, samples were diluted to the same protein concentrations with Laemmli sample buffer (Bio-Rad; Hercules, CA) and heated at 95°C for 7 min. Premature and mature SREBP1 proteins were separated by electrophoresis using 12% polyarcylamide gels (Cambrex Corporation, East Rutherford, NJ). Proteins were transferred to a PVDF membrane using a Bio-Rad trans-blot SD semi-dry transfer cell (Bio-Rad; Hercules, CA). Membranes were blocked in blocking buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween and 5% dried nonfat milk) on a rocker. Primary anti-SREBP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:750 in blocking buffer. Membranes were then washed in blocking buffer. Following wash, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer at 1:1500. Proteins were detected using ECL-Plus chemiluminescence substrate (Amersham Biosciences; Pittsburg, PA) according to manufacturer's instructions. Chemiluminescence was measured using a Chemidoc XRS digital imaging system and densitometry was quantified using Quantity One software (Bio-Rad; Hercules, CA).

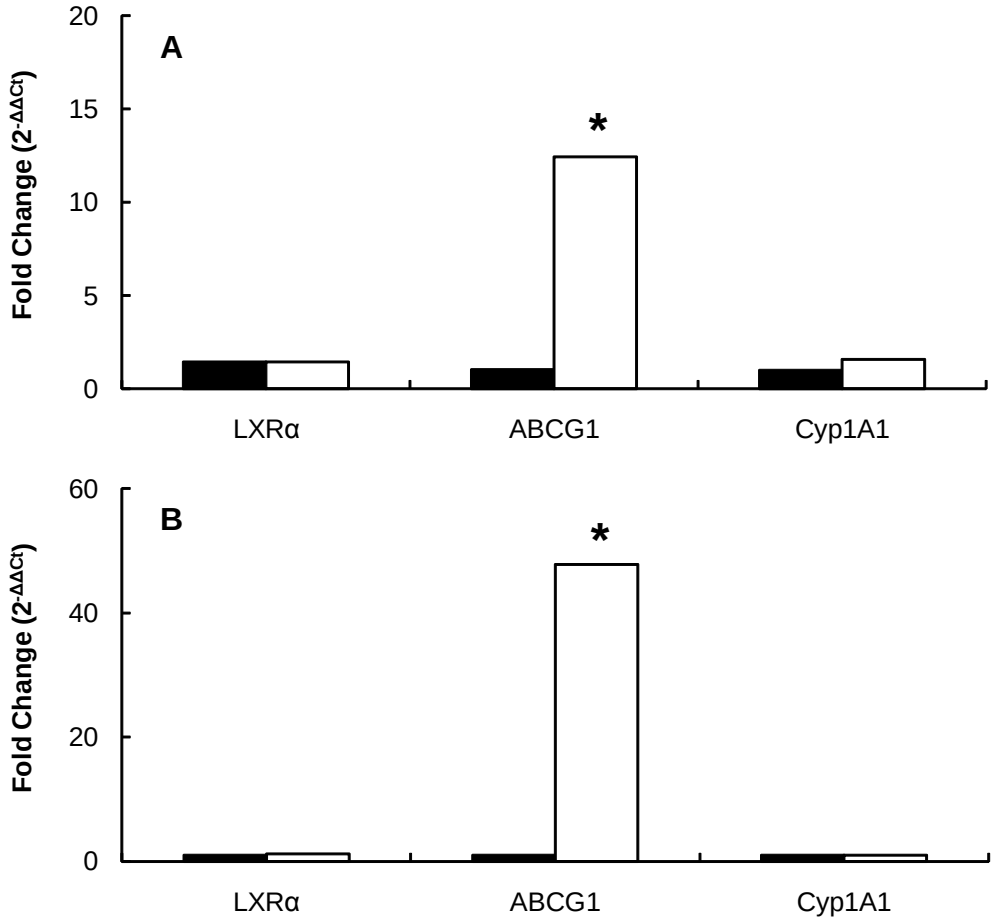
Statistical analysis

Data are reported as least squares means ± SEM unless otherwise noted. All data were analyzed using the Mixed procedure of SAS (SAS for Windows Version 9.1, SAS Institute Inc., Cary, NC). When analyzing the abundance of pSREBP1 or mSREBP1 in BME-UV, the model included the fixed effects of treatment and set. For analysis of all other data, the model included the fixed effects of treatment, set, and treatment by set interaction. Replicate within set was the random effect. Treatments include the presence of T09, GGPP, and the presence of fatty acids. One set represents one independent experiment. If a significant treatment effect was observed, Tukey's multiple comparison procedure was used to separate treatment means. Real-time PCR data were analyzed using original ΔCt values normalized with β-actin as the endogenous control gene. For the purpose of presentation, least squares means are illustrated as fold change ($2^{-\Delta\Delta Ct}$) relative to control.

RESULTS

Liver X receptor is a nuclear receptor capable of regulating the synthesis of fatty acids. The LXR agonist, T09, is used to activate LXR without modifying the expression of LXR (Houck et al., 2004). Comparable to published data, the mRNA expression of LXRα was unaffected by chronic treatment with T09 (Figure 4.1). The mRNA expression of several LXR target genes can be evaluated to assess the efficacy of T09 in activating LXR. Acute and chronic treatment of BME-UV with T09 dramatically increased the mRNA expression of ABCG1; however, expression of Cyp1A1 remained unaffected (Figure 4.1).

Figure 4.1. Effect of treating bovine mammary epithelial cells (BME-UV) with or without T09 (2 μM) for 8 (acute) or 24 h (chronic) on liver X receptor-α (LXRα) target gene expression. (A) Acute and (B) chronic effects of T09 on gene expression. Genes evaluated were liver X receptor-α (LXRα), ATP-binding cassette transporter-G1 (ABCG1), and cytochrome P4501A1 (Cyp1A1). Solid and open bars represent control and T09 treatments, respectively. Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. An asterisk indicates a significant difference in ΔCt from control, $P < 0.0001$.



Acute treatment of BME-UV with T09 significantly increased the incorporation of acetate into lipid (Figure 4.2). Similarly, chronic treatment of BME-UV with T09 for 24 h significantly increased acetate incorporation (Figure 4.3). Geranylgeranyl pyrophosphate, a LXR antagonist, inhibits the transcription of LXR target genes (Gan et al., 2001). Incubation of BME-UV with GGPP failed to reverse the T09 induced increase in acetate incorporation into fatty acids (Figure 4.3). Surprisingly, GGPP significantly increased *de novo* fatty acid synthesis in BME-UV when incubated alone or in combination with T09 (Figure 4.3).

Liver X receptor can regulate the transcription of lipogenic genes. In the present study, acute treatment of BME-UV with T09 significantly increased the mRNA expression of FAS and SREBP1 (Figure 4.4). However, the mRNA expression of INSIG1 and INSIG2 was unaffected by acute treatment of T09 (Figure 4.4). Compared to the acute treatment of BME-UV with T09, chronic treatment resulted in a greater increase in the mRNA expression of FAS and SREBP1 (Figure 4.4). Interestingly, the mRNA expression of INSIG1, a SREBP1 regulatory protein, was also enhanced in response to chronic T09 (Figure 4.4). The mRNA expression of INSIG2 was unaffected by chronic treatment with T09 (Figure 4.4).

Sterol regulatory element binding protein-1 is responsive to LXR activation. Acute incubation of BME-UV with T09 significantly increased the premature and mature forms of SREBP1 (Figure 4.5). *In vivo* and *in vitro, trans*-10, *cis*-12 CLA-induced decreases in *de novo* fatty acid synthesis are often in response to a decrease in SREBP1 protein abundance. In addition, fatty acids varying in degree of saturation inhibit fatty acid synthesis in cell culture. Incubation of BME-UV with T09 failed to

Figure 4.2. Effect of treating bovine mammary epithelial cells (BME-UV) with or without T09 (2 μM) for 8 h on *de novo* fatty acid synthesis. Error bars represent SEM for two independent experiments with three replicates per experiment. An asterisk indicates a significant difference from control, $P < 0.0001$.

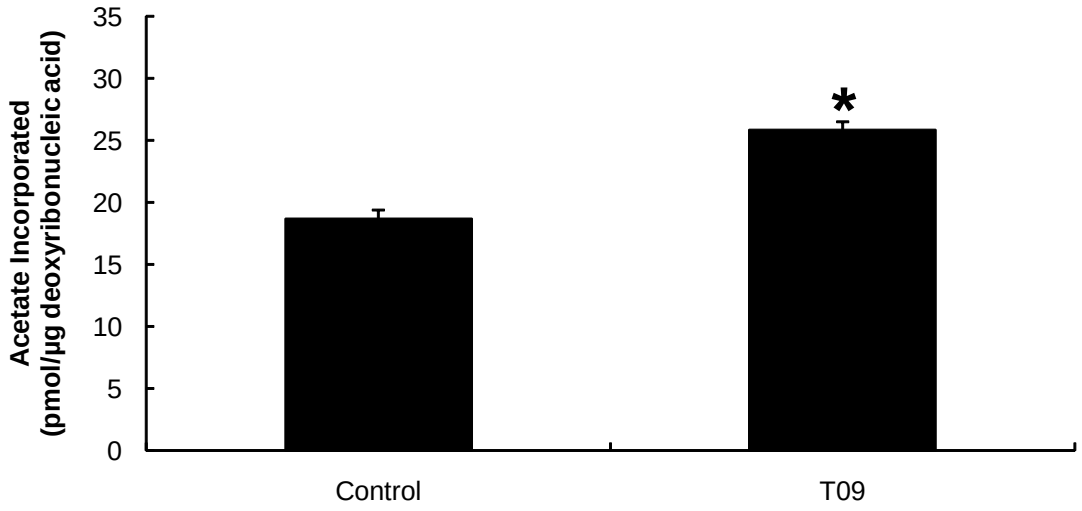


Figure 4.3. Effect of treating bovine mammary epithelial cells (BME-UV) with T09 (2 µM), GGPP (10 µM), or both for 24 h on *de novo* fatty acid synthesis. Control represents the absence of T09 and GGPP. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences are signified by differing superscripts, $P < 0.0001$.

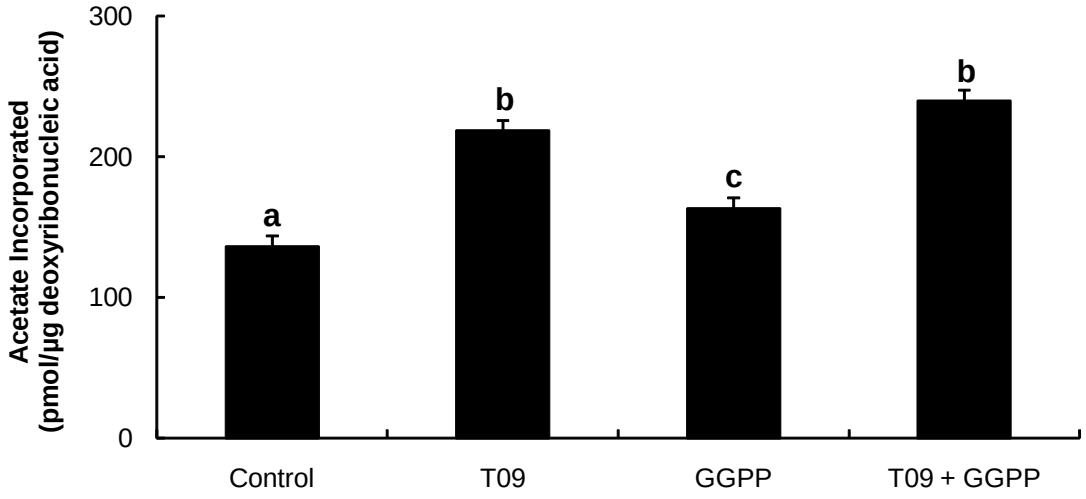


Figure 4.4. Effect of treating bovine mammary epithelial cells (BME-UV) with or without T09 (2 μM) for 8 (acute) or 24 h (chronic) on gene expression. (A) Acute and (B) chronic effects of T09 on lipogenic gene expression. Lipogenic genes evaluated were fatty acid synthase (FAS), sterol regulatory element binding protein-1 (SREBP1), insulin-induced gene-1 (INSIG1), and insulin-induced gene-2 (INSIG2). Solid and open bars represent control and T09 treatments, respectively. Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. An asterisk indicates a significant difference in ΔCt from control, $P < 0.001$.

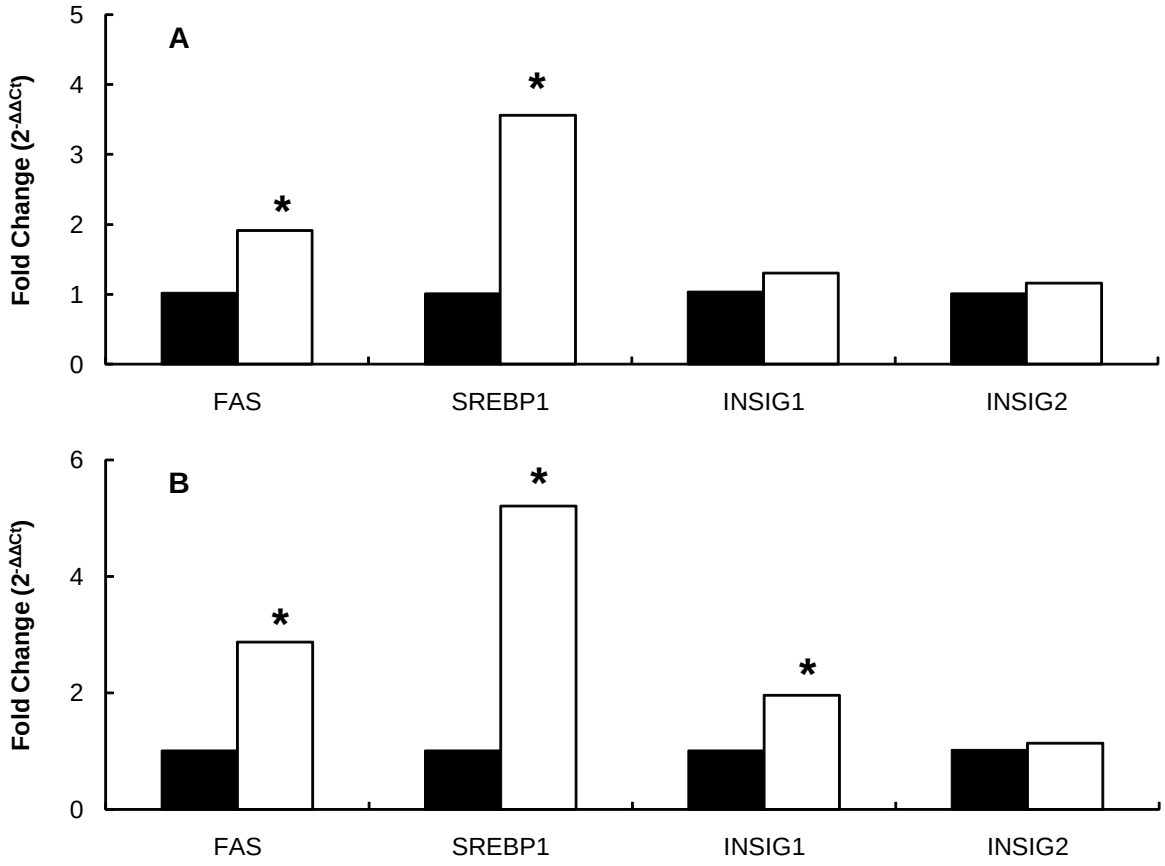
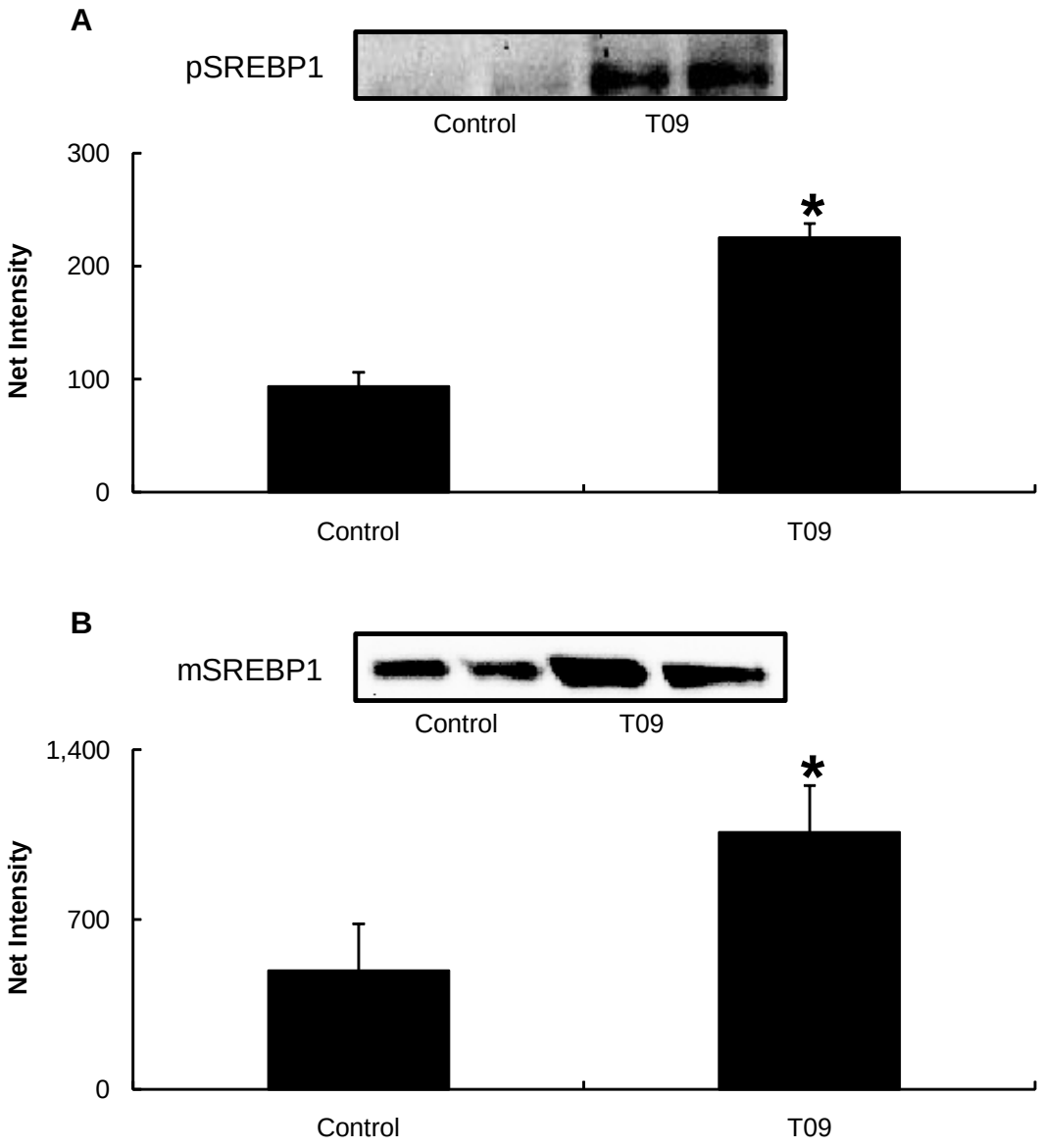


Figure 4.5. Effect of treating bovine mammary epithelial cells (BME-UV) with or without T09 (2 μM) for 8 h on premature sterol regulatory element binding protein-1 (pSREBP1) and mature sterol regulatory element binding protein-1 (mSREBP1) protein abundance. (A) Effects of treatment on pSREBP1 protein abundance. (B) Effects of treatment on mSREBP1 protein abundance. Error bars represent SEM for two independent experiments. An asterisk indicates a significant difference from control, (A) $P < 0.05$ and (B) $P < 0.10$.



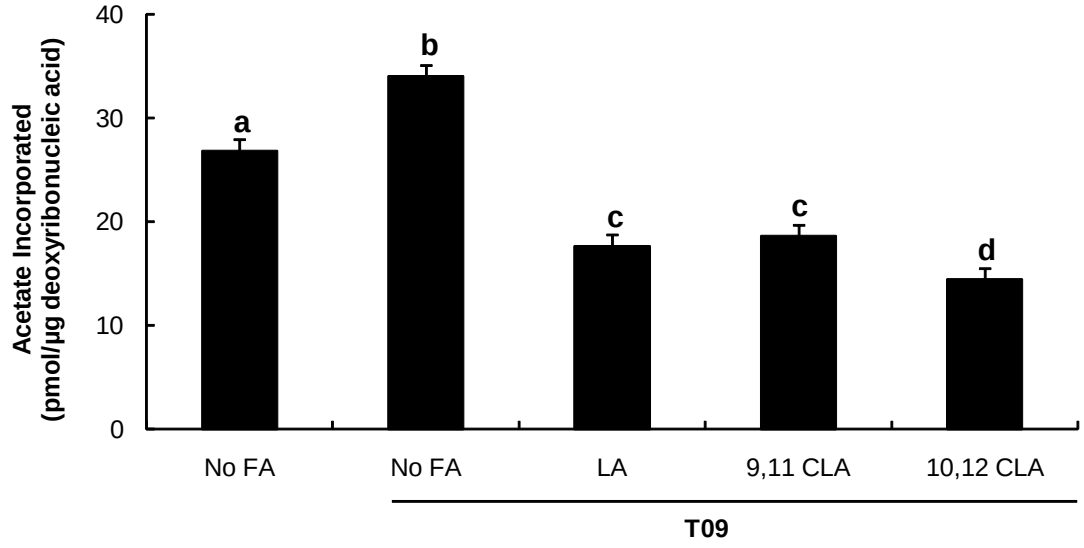
reverse the inhibitory effect of polyunsaturated fatty acids on *de novo* fatty acid synthesis (Figure 4.6).

DISCUSSION

The function of LXRs in the mammary gland is undefined. In dairy cows, LXRα, but not LXRβ expression is increased during lactation compared to nonlactating mammary tissue (Harvatine and Bauman, 2007). To our knowledge, the activation of LXRα in BME-UV has not been investigated prior to this experiment.

Nuclear receptors such as LXR undergo a conformational change upon ligand binding enhancing their activity. T0901317 is a synthetic nonsteroidal compound and a highly potent, selective nonsteroidal LXRα ligand (Schultz et al., 2000). In addition, T09 is capable of increasing LXR activity without modifying LXR expression (Houck et al., 2004). Similarly, LXRα mRNA expression was unaffected by acute or chronic treatment with T09 in BME-UV. ATP-binding cassette transporter-G1 and Cyp1A1 are known LXR target genes and are upregulated in response to T09 (Dressel et al., 2003; Westerink and Schoonen, 2007). ATP-binding cassette transporter-G1 has been implicated in the efflux of cholesterol to high density lipoprotein (Wang et al., 2004). Recently, the presence of ABCG1 has been verified in the bovine mammary gland (Farke et al., 2008). In response to acute and chronic treatment with T09, ABCG1 mRNA expression increased 11- and 43-fold, respectively. Cytochrome P4501A1 was unresponsive to T09. We can conclude that T09 is capable of affecting the expression of ABCG1, a primary target gene, without influencing LXRα mRNA expression therefore

Figure 4.6. Effect of treating bovine mammary epithelial cells (BME-UV) with T09 (2 µM) and fatty acids (50 µM) on *de novo* fatty acid synthesis. Cells were incubated with T09 and fatty acids 8 and 24 h prior to addition of radio-labeled acetate, respectively. Fatty acids included linoleic acid (LA), *cis*-9, *trans*-11 conjugated LA (9,11 CLA), or *trans*-10, *cis*-12 CLA (10,12 CLA). Cells incubated in the absence of fatty acids (No FA) and T09 represented the control. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences are signified by differing superscripts, $P < 0.0001$.



T09 is an effective LXR agonist in BME-UV. In addition, LXR activation may serve as a potential regulator of cholesterol homeostasis in the bovine mammary gland.

Liver X receptors regulate the synthesis of lipid including cholesterol, bile acids, and fatty acids (see review by Edwards et al. (2002)). Liver X receptor-α is a major sensor of dietary cholesterol and functions as an important transcriptional control point in bile acid synthesis (Peet et al., 1998b). The ability of LXR to regulate fatty acid synthesis has also been evaluated. Peet and coworkers (1998b) discovered that mice carrying a targeted disruption in the LXRα gene had decreased expression of ACC, FAS, steroyl-CoA desaturase-1, and SREBP1. Furthermore, oral administration of T09 to mice resulted in the upregulation of lipogenic enzyme expression and increased plasma triacylglycerol levels (Schultz et al., 2000). In the present study, acute and chronic treatment with 2 µM T09 significantly increased *de novo* fatty acid synthesis in BME-UV by 38% and 60%, respectively. Treatment of BME-UV with T09 concentrations greater that 2 µM resulted in significant cell death (data not shown). Geranylgeranyl pyrophosphate is a LXR antagonist and can decrease the transcription of LXR target genes (Gan et al., 2001; Argmann et al., 2005). Chronic treatment of BME-UV with GGPP failed to reverse the T09-induced increase in *de novo* fatty acid synthesis.

Activation of LXR by T09 enhances the expression of SREBP1 (Repa et al., 2000; Montanaro et al., 2007). Repa and coworkers (2000) concluded that cholesterol-derived oxysterols activate LXR to induce expression of the mouse SREBP1c gene through a LXRE located within its proximal promoter. In addition, the SREBP1a and SREBP2 isoforms are unresponsive to LXR activation by T09 (Schultz et al., 2000;

DeBose-Boyd et al., 2001). The expression of SREBP1 has been shown to be significantly upregulated during lactation in mice and cows and is considered the primary regulator of milk fat synthesis (Anderson et al., 2007; Rudolph et al., 2007; Bionaz and Loor, 2008b). In the present study, acute and chronic treatment of BME-UV with T09 increased the mRNA expression of SREBP1 255% and 416%, respectively. We also observed a significant increase in protein abundance for the premature and mature forms of SREBP1. Insulin-induced gene-1 and INSIG2 are integral membrane proteins that cause retention of SREBP1 in the endoplasmic reticulum, preventing SREBP1 activation. In the present study, chronic treatment of BME-UV with T09 increased the mRNA expression of INSIG1. Interestingly, the INSIG1 promoter can be regulated by transcriptionally active SREBP1 (Kast-Woelbern et al., 2004). Overexpression of INSIG1 in the livers of transgenic mice inhibits SREBP1 processing and reduces insulin-stimulated lipogenesis (Engelking et al., 2004). In the current study, it is possible that INSIG1 retained a portion of pSREBP1 within the endoplasmic reticulum.

Fatty acid synthase is a known SREBP1 target gene and an essential enzyme in the *de novo* synthesis of fatty acids (Magana and Osborne, 1996; Harvatine and Bauman, 2006). In human preadipocytes, the mRNA expression of FAS and SREBP1c is increased in response to T09 (Darimont et al., 2006). In the present study, acute and chronic treatment with T09 increased FAS mRNA expression by 89% and 185%, respectively. Liver X receptors regulate fatty acid synthesis by increasing the transcription of FAS via indirect and direct mechanisms. Enhanced expression of SREBP1 in response to T09 most likely increased FAS expression indirectly. However,

Joseph and coworkers (2002) demonstrated that in addition to tandem SREBP1c sites, the FAS promoter contains a binding site for the LXR/RXR heterodimers. Joseph and coworkers (2002) confirmed that maximum induction of FAS requires both LXR and SREBP1c binding to their respective response elements within the promoter region. Therefore, it is possible that LXR activation in bovine mammary epithelial cells may regulate FAS gene expression via indirect and direct mechanisms. Further examination of the activation of the FAS promoter will be required to evaluate this hypothesis. Additionally, bioinformatic examination of the promoters of other lipogenic genes in the bovine genome may reveal liver X receptor response element (LXRE) sites that regulate transcription. We can conclude that the dramatic increase in SREBP1 and FAS expression most likely contributed to the significant increase in acetate incorporation in response to acute and chronic treatment with T09.

The effects of *trans*-10, *cis*-12 CLA on lipogenic gene expression in the bovine mammary gland have been well established (Baumgard et al., 2000; Harvatine and Bauman, 2006). Sterol regulatory element binding protein-1 is a known regulator of milk fat synthesis; therefore, it has been proposed that SREBP1 may be responsible for regulating their expression in response to *trans*-10, *cis*-12 CLA (Peterson et al., 2004; Harvatine et al., 2009). In support of this theory, the mRNA expression of SREBP1 is significantly decreased in response to *trans*-10, *cis*-12 CLA treatment *in vivo* (Harvatine and Bauman, 2006). *In vitro,* Peterson and coworkers (2004) observed a similar decrease in the mRNA expression of SREBP1 in response to *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA treatments compared to a bovine serum albumin control. In the present study, BME-UV cells treated with LA, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12

CLA for 24 h significantly decreased acetate incorporation into fatty acids by 34%, 31%, and 46%, respectively. Treatment of BME-UV with T09 for 8 h failed to reverse the inhibitory effect of polyunsaturated fatty acids on *de novo* fatty acid synthesis. Recent studies may suggest that polyunsaturated fatty acids suppress SREBP1c gene expression by inhibiting LXR/RXR heterodimers binding to LXRE found within the promoter region SREBP1c (Ou et al., 2001; Yoshikawa et al., 2002). It is possible that the concentration of fatty acid was high enough to prevent the LXR/RXR heterodimers from binding to the LXRE. If the concentration of fatty acid was lower, T09 may have been able to reverse the inhibitory effect of polyunsaturated fatty acids on *de novo* fatty acid synthesis. The ability of unsaturated fatty acids to accelerate SREBP1 mRNA degradation has been documented and may explain our observed effects (Xu et al., 2001). Activation of LXR may have upregulated the expression of SREBP1; however, the presence of polyunsaturated fatty acid may have accelerated its degradation.

In conclusion, LXR regulates the *de novo* synthesis of fatty acids in BME-UV by promoting the transcription of SREBP1 and FAS. We also propose the inhibition of *de novo* fatty acid synthesis by polyunsaturated fatty acids cannot be reversed by LXR activation. Activation of LXR by T09 may prove to be a useful tool in identifying the relative contributions of LXRα and SREBP1 towards the overall synthesis of fatty acids in the bovine mammary gland.

CHAPTER 5

Activation of AMP-Activated Protein Kinase Inhibits *de novo* Fatty Acid Synthesis in Bovine Mammary Epithelial Cells

INTRODUCTION

AMP-activated protein kinase (AMPK) plays an integral role in monitoring intracellular energy status and regulating the uptake and metabolism of glucose and fatty acids as well as the synthesis and oxidation of fatty acids, cholesterol, glycogen, and protein to meet energy demand. AMP-activated protein kinase is activated by increases in ATP utilization or decreases in ATP production caused by exercise, prolonged fasting, and/or hypoxia (Stephens et al., 2002; Zou et al., 2003; Minokoshi et al., 2004). In addition to increases in the AMP to ATP ratio, AMPK is activated by rises in intracellular Ca^{2+} (Hurley et al., 2005; Woods et al., 2005). Rises in intracellular Ca^{2+} can be caused by inositol triphosphate produced by phospholipase C coupled to the G protein Gq (Kishi et al., 2000).

AMP-activated protein kinase is a heterotrimeric protein composed of one catalytic (α) and two regulatory subunits (β and γ). Each subunit has more than one isoform derived from a specific gene; therefore, twelve possible heterotimeric combinations may exist. The known AMPK isoforms include α1, α2, β1, β2, γ1, γ2, and γ3 (Thornton et al., 1998; Stephens et al., 2002; Barnes et al., 2004). The AMPK complex becomes activated when AMP binds to Bateman domains on the γ subunit resulting in a conformational change permitting the phosphorylation of a threonine residue (Thr-172) on the α subunit. Upstream kinases that phosphorylate the α subunit following conformational change include serine/threonine kinase 11 (LKB1) and Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK). Serine/threonine kinase 11 is

considered the major upstream AMPK kinase responsible for increasing energy supply by sensing increases in the AMP to ATP ratio (Woods et al., 2003; Lizcano et al., 2004). Ca^{2+}/calmodulin-dependent kinase kinase is activated by increases in intracellular Ca^{2+} concentration (Hawley et al., 2005; Woods et al., 2005). Ca^{2+}/calmodulin-dependent kinase kinase-β, one of two known isoforms of CaMKK, directly activates AMPK in HeLa cells (Hurley et al., 2005) as well as C2C12 myotubes (Shen et al., 2007).

The ability of AMPK to regulate lipid metabolism has multiple dimensions. 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) activates AMPK which phosphorylates and inactivates acetyl-CoA carboxylase (ACC; Park et al., 2002a). Acetyl-CoA carboxylase is the rate limiting enzyme of *de novo* fatty acid synthesis in the mammary gland of the lactating dairy cow (Wakil et al., 1983). Activation of AMPK has also been shown to downregulate glycerol-3-phosphate acyltransferase (GPAT), an enzyme necessary for triacylglycerol (TAG) synthesis (Muoio et al., 1999). Interestingly, AMPK activation may also regulate the transcription of lipogenic transcription factors in addition to post-translationally modifying lipogenic enzymes. Activation of AMPK by Metformin can decrease the expression of lipogenic genes including sterol regulatory element-binding protein-1 (SREBP1) and ACCα in rat hepatocytes (Zhou et al., 2001). Fortez and coworkers (1998) observed decreases in fatty acid synthase (FAS) gene expression in response to AMPK activation.

Milk fat from cows is primarily composed of TAG and is the most variable component of milk (see review by (Palmquist, 2006)). Acetyl-CoA carboxylase, FAS, and GPAT are three lipogenic enzymes required for milk fat production (Palmquist, 2006). In ruminants, these enzymes can be downregulated in response to restricted

dietary intake (Ingle et al., 1973; Bauman et al., 2006). Since milk fat synthesis represents a significant energy expenditure for the cow, AMPK may regulate milk fat synthesis, an energy consuming process, to modulate mammary epithelial cell energy utilization. Little is known about the role of AMPK in the lactating dairy cow. The objectives of the present study were to determine the mRNA expression of α, β, and γ subunits of AMPK in various bovine tissues and evaluate the effect of AMPK activation on *de novo* fatty acid synthesis in bovine mammary epithelial cells.

MATERIALS AND METHODS

To evaluate the tissue distribution of AMPK, adipose, liver, and muscle tissue samples were harvested from three bulls killed at the Department of Food Science and Technology, Virginia Tech. Mammary tissue was harvested from two Holstein cows and one Jersey cow euthanized at the College of Veterinary Medicine, Virginia Tech, for orthopedic injuries. Tissues were compared with cells from the MAC-T bovine mammary epithelial cell line (Huynh et al., 1991). All tissues collected at slaughter were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Cell culture and treatments

All experiments utilized the MAC-T bovine mammary epithelial cell line. Cells were routinely cultured at 37°C in 5% CO_2, and the medium was changed every 24 h throughout the duration of the experiment. Cells were seeded on plastic cell culture plates at a density of 2.6×10^4 cells/cm^2. Cells were grown in basal medium (Dulbecco's Modified Eagle's Medium (DMEM) with 10 kU/ml penicillin, 10 mg/ml

streptomycin, and 25 µg/ml amphotericin B) supplemented with 10% fetal bovine serum. Once cells reached confluence (approximately 72 h post seeding), serum was removed and hormones (0.1 µg/mL insulin and 1.5 µg/mL prolactin; Sigma Chemical Co., St. Louis, MO) were added to the basal media. Cells were cultured in basal media with hormones for 24 h and then treatments were applied.

Cells were treated with AICAR (200, 400, or 600 µM; Sigma Chemical Co., St. Louis, MO) for 1, 4, or 24 h. In a separate experiment, cells were treated with ionomycin (1 µM; Sigma Chemical Co., St. Louis, MO) for 1 h. Dimethyl sulfoxide (DMSO; Sigma Chemicals Co., St. Louis, MO) served as control when evaluating the effects of ionomycin. Cells were treated with no more than 0.23% DMSO. To evaluate the effects of energy removal, cells were incubated with DMEM without glucose for 4 h. Cells incubated with DMEM with glucose served as control.

Fatty acid synthesis assay

De novo fatty acid synthesis was determined by quantifying the incorporation of [1-^{14}C]-labeled acetate into lipid. Methods were adapted from Peterson and coworkers (2004) and modified as described. Cells were cultured in 12-well cell culture plates and incubated with [1-^{14}C]-acetate (MP Biomedicals, Solon, OH) for a period of 4 h. After 1 or 20 h of AICAR treatment, cells were cultured in 3 mM acetate (0.37 µCi/µmol). Following the 4 h incubation with [1-^{14}C]-labeled acetate, cells were lysed in wells with sodium dodecyl sulfate buffer (0.1% in phosphate buffered saline). Lipid within the lysate was then extracted using hexane:isopropanol (3:2). The solvent layer was combined with 16 ml of scintillation cocktail (Scintisafe 30% Cocktail; Fisher Scientific,

Pittsburgh, PA) for quantification of label incorporation into lipid using a LS 6000LL Beckmann scintillation counter. Activity was calculated and expressed as pmol of acetate incorporated per µg of DNA.

Real time PCR

Total RNA was extracted from snap-frozen tissues using TRI Reagent (1 ml/50-100 mg tissue; Molecular Research Center, Cincinnati, OH). Cultured cells were lysed in 6-well culture plates using 1 mL of TRI Reagent (Molecular Research Center Inc.; Cincinnati, OH) per well. Total RNA was isolated according to manufacturer's instructions. Ribonucleic acid pellets were resuspended in RNase-free water, and quantified at 260 nm using a spectrophotometer. Total RNA was reverse transcribed (500 ng per reaction) into cDNA using the Omniscript reverse transcription kit (Qiagen; Valencia, CA) according to manufacturer's instructions using oligo(dT) (Roche Applied Science; Indianapolis, IN) as the primer.

Real-time PCR reactions were performed using the Quantitect SYBR Green PCR kit (Qiagen; Valencia, CA) and an Applied Biosystems 7300 Real-time PCR machine (Applied Biosystems; Foster City, CA). Quantification of gene transcripts for ACCα, FAS, lipoprotein lipase (LPL), fatty acid binding protein-3 (FABP3), GPAT, 1-acylglycerol-3-phosphate acyltransferase-6 (AGPAT), peroxisome-proliferator activated receptor-γ (PPARγ), liver X receptor-α (LXRα), SREBP1, α1 AMPK, α2 AMPK, β1 AMPK, β2 AMPK, γ1 AMPK, γ2 AMPK, and γ3 AMPK was completed using gene-specific primers (Table 5.1). β-actin was used as the endogenous control gene, and adipose was used as the calibrator for making relative comparisons between tissues

Table 5.1. Primer sequences used to detect ACCα, FAS, LPL, FABP3, GPAT, AGPAT, PPARγ, LXRα, SREBP1, α AMPK, β AMPK, γ AMPK and β-actin[1].

Target Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')	Product Size (bp)
ACCα	GGGTGAAAGACTGGGTTGGAA	GTAGTGTAGGCACGAGACAG	172
FAS	AATGACCACTTTGCCGATGT	TGAAGGACGGTGTACCACAA	152
LPL	GAGCCAAAAGAAGCAGCAAG	TGTAGGGAAAATGGGACGGA	181
FABP3	GAACTCGACTCCCAGCTTGAA	GAAGCTACTAACACCATCCGAA	101
GPAT	ATTGACCCTTGGCACGATAG	GAAACACCCTTCCACGACAA	187
AGPAT	AAGCAAGTTGCCCATCCTCA	AGCTTTAACCTCGGTGTCAAA	100
PPARγ	CATCTTCCAGGGGTGTCAGT	TCCTACCCCAGGAGTATAGG	186
LXRα	TCAACCCCATCTTCGAGTTC	ACGACTACTTTGACCACTCG	232
SREBP1[2]	ATGCCATCGAGAAACGCTAC	CTCTTGGACTCAGACGCCTG	180
α1 AMPK	GAGCTTGCCAAAGGAATGAT	AACTCTACACGCGCTTAGAT	158
α2 AMPK	ACAGCCCTAAAGCACGATGT	GTAGAACCTTAGGCTTCAGTT	101
β1AMPK	CGATCTGGAAGTGAACGACA	AGACCCAGGAAATTGTTGACC	106
β2 AMPK	AGGATTTGGAGGACTCCGTA	CTCGTGGTTCTAAGGTGACT	126
γ1 AMPK	GCTGTATGGAGGAGGCTGAG	CCAATAACGAAAAGGACCGT	173
γ2 AMPK	CTCTTCGATGCTGTGCACTCG	AGGAGTTCAAGGAGGTCGAA	123
γ3 AMPK	TAGAGTTCTCAGCCCCAGCA	CCAGATGTACGTGAAGTACGT	154
β-actin	CTCTTCCAGCCTTCCTTCCT	CGTCTTTCTCTAGTGACGGG	178

[1]ACCα, acetyl-CoA carboxylase; FAS, fatty acid synthase; LPL, lipoprotein lipase; FABP3, fatty acid binding protein-3; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerol phosphate acyltransferase; PPARγ, peroxisome proliferator-activated receptor-γ; LXRα, liver X receptor-α; SREBP1, sterol regulatory element binding protein-1; AMPK, AMP-activated protein kinase.

[2]Primer pairs do not distinguish between SREBP1a and SREBP1c isoforms.

within each AMPK isoform. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Reaction conditions were as follows: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Each reaction was performed in duplicate wells.

DNA quantification

Cells were harvested in DNA assay buffer (Tris Base, EDTA, NaCl; pH 7.4) then sonicated (2 × 5 s) on ice. Deoxyribonucleic acid concentration of cell lysates was assayed as described by Labarca and Paigen (1980) with the following modifications. Briefly, 2 µL of cell lysate was transferred to a cuvette followed by 1998 µL of DNA assay buffer to yield a total assay volume of 2 mL. Samples were measured in triplicate using a DyNA Quant 200 fluoremeter (Hoefer Pharmacia Biotech; San Francisco, CA). Calf thymus DNA (Sigma Chemical Co.; St. Louis, MO) was used as a standard.

Immunoblotting

Cells were seeded on 6-well cell culture plates. In one well, ice-cold phosphate buffered saline with phosphatase inhibitors (5 mM NaF and 1 mM Na_3VO_4) was used to wash cells prior to harvest. Cells were harvested in ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA, and protease inhibitor cocktail) with phosphatase inhibitors for 15 min on ice. Lysate was centrifuged at 14,000 × *g* at 4°C for 10 min. Pellets were discarded and supernatants were assayed for protein concentration using the Bradford assay (Bio-Rad; Hercules, CA). To ensure equal loading, samples were diluted to the same protein concentrations with Laemmli sample

buffer (Bio-Rad; Hercules, CA) and heated at 95°C for 7 min. Proteins were separated by electrophoresis using 7.5% polyarcylamide gels (Cambrex Corporation, East Rutherford, NJ) and transferred to a PVDF membrane using a Bio-Rad trans-blot SD semi-dry transfer cell (Bio-Rad; Hercules, CA). Membranes were blocked in blocking buffer (0.05 M Tris pH 7.4, 0.2M NaCl, 0.1% Tween, and 5% dried nonfat milk) on a rocker. Primary anti-ACC or anti-phospho-ACC antibodies (Cell Signaling Technology; Beverly, MA) were applied at 1:1000 in blocking buffer. Membranes were then washed in blocking buffer. Following washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer at 1:2000. Proteins were detected using ECL-Plus chemiluminescence substrate (Amersham Biosciences; Pittsburg, PA) according to manufacturer's instructions. Chemiluminescence was measured using a Chemidoc XRS digital imaging system and densitometry was quantified using Quantity One software (Bio-Rad; Hercules, CA).

Acetyl-CoA carboxylase activity assay

Bovine mammary epithelial cells were seeded on 100-mm cell culture dishes and treated with AICAR for 1 h. After treatment, ice-cold phosphate buffered saline with phosphatase inhibitors (5 mM NaF and 1 mM Na_3VO_4) was used to wash cells prior to harvest. Cells were harvested in ice-cold lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA, and protease inhibitor cocktail) with phosphatase inhibitors for 15 min on ice. Lysate was centrifuged at 14,000 × *g* at 4°C for 10 min.

Pellets were discarded and supernatants were assayed for protein concentration using the Bradford assay.

Activity of ACC in MAC-T was determined by quantifying the incorporation of ^{14}C-labeled $NaH^{14}CO_3$ into acid-stable malonyl-CoA. Methods were adapted from Chang and coworkers (1967) and modified as described. Samples were first pre-incubated at 37°C for 30 min. The pre-incubation reaction composition was 100 mM Tris-HCl pH 7.3, 200 mM sodium citrate, 200 mM $MgCl_2$, 2 mM glutathione, and 8 mg/mL bovine serum albumin, in a final volume of 0.5 mL. Following pre-incubation, reactions were initiated by the addition of 0.5 mL of assay mixture containing 100 mM Tris-HCl pH 7.3, 7.8 mM ATP, 0.4 mM acetyl-CoA, 2 mM glutathione, and 5 mM $NaH^{14}CO_3$ (1.25 µCi/mL). Reactions were allowed to proceed for 45 minutes at 37°C and then terminated with 0.25 mL of 6 N HCl followed by evolution of unincorporated $^{14}CO_2$. Scintillation cocktail was added and quantification of label into malonyl-CoA was determined using a LS 6000LL Beckmann scintillation counter. Activity was calculated and expressed as nmol of $NaH^{14}CO_3$ incorporated per mg of protein.

Statistical analysis

Data are reported as least squares means ± SEM unless otherwise noted. All data were analyzed using the Mixed procedure of SAS (SAS for Windows Version 9.1, SAS Institute Inc., Cary, NC). When analyzing the presence of AMPK subunits in bovine tissues and MAC-T, the model included tissues or cells and animal or plate of cells. Treatments included adipose, liver, muscle, and mammary tissue in addition to MAC-T. One sample represents one animal or one well of plated MAC-T. When

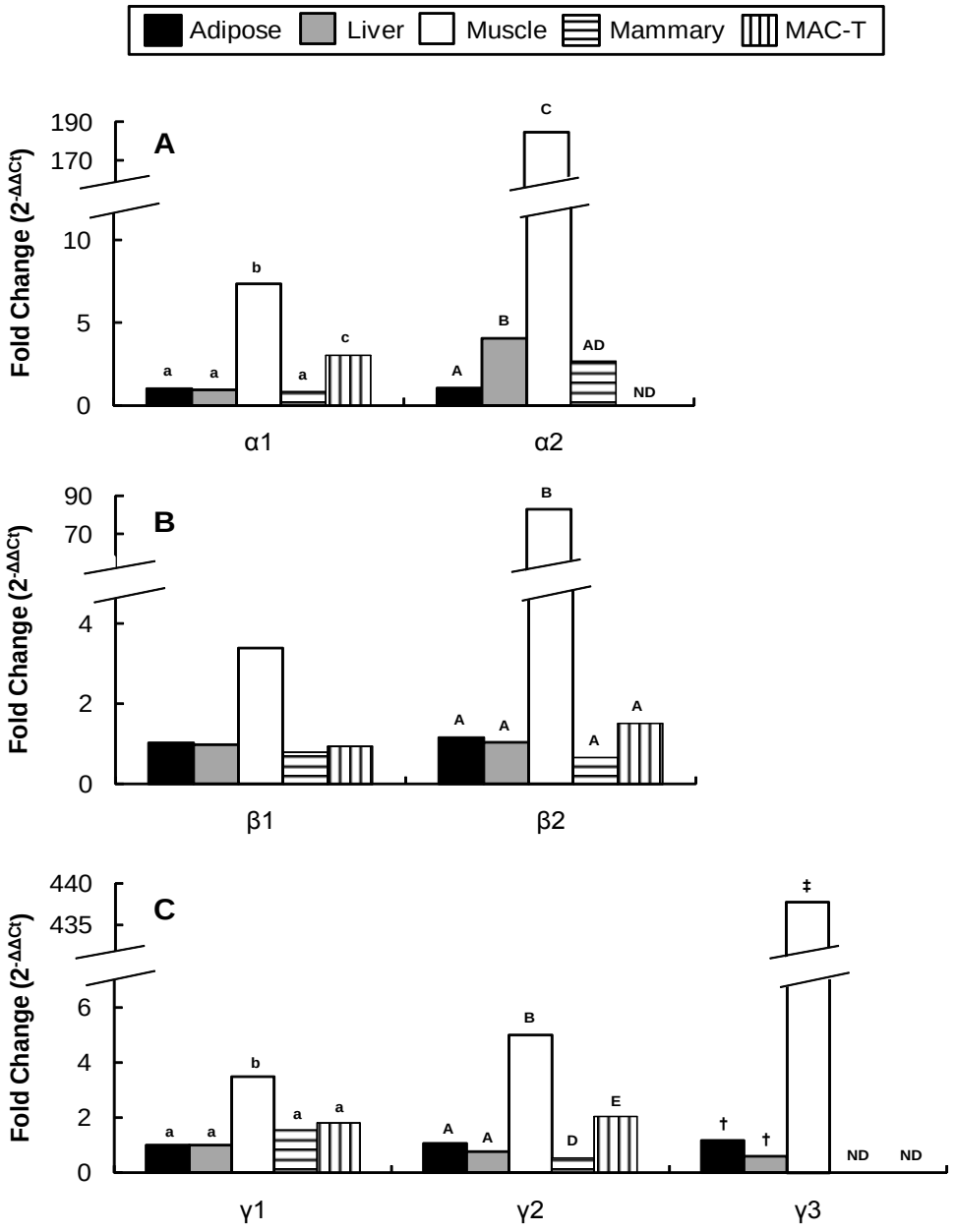
analyzing the activity of ACC in MAC-T, the model included the fixed effects of treatment and set. For analysis of all other data, the model included the fixed effects of treatment, set, and treatment by set interaction. Replicate within set was the random effect. Treatments include the presence of AICAR, the presence of glucose, or the presence of ionomycin. One set represents one independent experiment. If a significant treatment effect was observed, Tukey's multiple comparison procedure was used to separate treatment means. Real-time PCR data were analyzed using original ΔCt values normalized with β-actin as the endogenous control gene. For the purpose of presentation, least squares means are illustrated as fold change ($2^{-\Delta\Delta Ct}$) relative to control

RESULTS

AMP-activated protein kinase is expressed in various tissues throughout the mammalian body. The relative mRNA expression of the known α, β, and γ isoforms of AMPK varies depending on tissue type. In the present study, α AMPK, β AMPK, and γ AMPK mRNA were found in all bovine tissues examined and the MAC-T bovine mammary epithelial cell line; however, the α2 isoform was not expressed in MAC-T nor was the γ3 isoform found in mammary tissue or MAC-T (Figure 5.1). For all three subunits of AMPK, the mRNA expression was greater in muscle compared to other bovine tissues and MAC-T.

Activation of AMPK occurs in response to an increase in the AMP:ATP ratio. The AMPK activator, AICAR, is routinely used to mimic this effect after activation to AICAR monophosphate (ZMP), an AMP analog (Corton et al., 1995). Acute treatment of MAC-

Figure 5.1. Bovine tissue and bovine mammary epithelial cell (MAC-T) presence of α AMP-activated protein kinase (AMPK), β AMPK, and γ AMPK mRNA. (A) Tissue and MAC-T presence of α1 and α2 isoforms. (B) Tissue and MAC-T presence of β1 and β2 isoforms. (C) Tissue and MAC-T presence of γ1, γ2, and γ3 isoforms. Real-time PCR was performed using RNA extracted from bull tissues (adipose, liver, muscle; *n* = 3), cow mammary tissue (*n* = 3), and MAC-T (*n* = 3). β-actin was used as the endogenous control gene, and adipose was used as the calibrator for making relative comparisons between tissues within each isoform. Data is illustrated as least squares means. Differences in ΔCt values for treatments are signified by differing superscripts within isoform, $P < 0.01$. Isoforms not detected are denoted as ND.



T with increasing concentrations of AICAR significantly decreased incorporation of acetate into lipid (Figure 5.2). Similarly, chronic treatment of MAC-T with 400 and 600 µM AICAR significantly decreased acetate incorporation compared to control; however, treatment with 200 µM AICAR significantly increased acetate incorporation (Figure 5.2).

AMP-activated protein kinase phosphorylates a variety of cellular proteins thereby stimulating or inhibiting their activity. The phosphorylation and subsequent inactivation of ACC by AMPK is widely observed in response to AICAR treatment (Merrill et al., 1997; Zhou et al., 2001; Park et al., 2002a). Bovine mammary epithelial cells incubated with AICAR for 1 h significantly increased the phosphorylation of ACCα (Figure 5.3). The phosphorylation of ACC by AMPK has been shown to be accompanied by a decrease in ACC activity (Munday et al., 1988). In response to increased phosphorylation of ACCα, we observed a significant decrease in ACC activity after treating MAC-T with AICAR for 1 h (Figure 5.4)

In addition to the short-term regulation of proteins by phosphorylation, AMPK may also exert long-term effects by controlling gene transcription. Chronic treatment of AICAR (600 µM) resulted in a significant decrease in the mRNA expression of LPL, GPAT, and PPARγ (Figure 5.5. and 5.6). However, chronic treatment of AICAR significantly increased the mRNA expression of FAS, FABP3, and SREBP1 (Figure 5.5 and 5.6). The mRNA expression of ACCα, AGPAT, and LXRα were unaffected by chronic treatment of AICAR.

AMP-activated protein kinase is able to detect decreases in energy supply, and reductions in glucose availability can dramatically alter the ratio of AMP:ATP, resulting in activation of AMPK (Salt et al., 1998). Incubation of MAC-T in the absence of

Figure 5.2. Effect of treating bovine mammary epithelial cells (MAC-T) with either 0 (control), 200, 400, or 600 µM AICAR for 4 (acute) or 24 h (chronic) on *de novo* fatty acid synthesis. Solid and open bars represent acute and chronic treatments, respectively. Error bars represent SEM for two independent experiments with three replicates per experiment. Treatment differences are signified by differing superscripts within acute (lowercase) or chronic (uppercase) treatments, $P < 0.0001$.

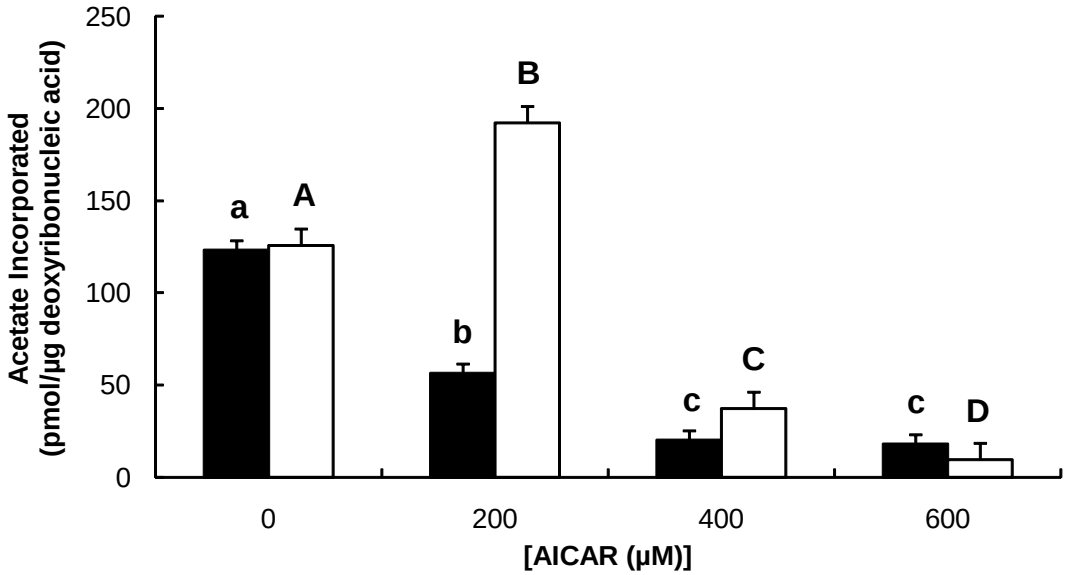


Figure 5.3. Effect of treating bovine mammary epithelial cells (MAC-T) with or without AICAR (600 μM) for 1 h on ratio of phosphorylated-ACCα (P-ACCα) to total ACCα protein abundance. Error bars represent SEM for two independent experiments with three replicates per experiment. An asterisk indicates a significant difference from control, $P < 0.0001$.

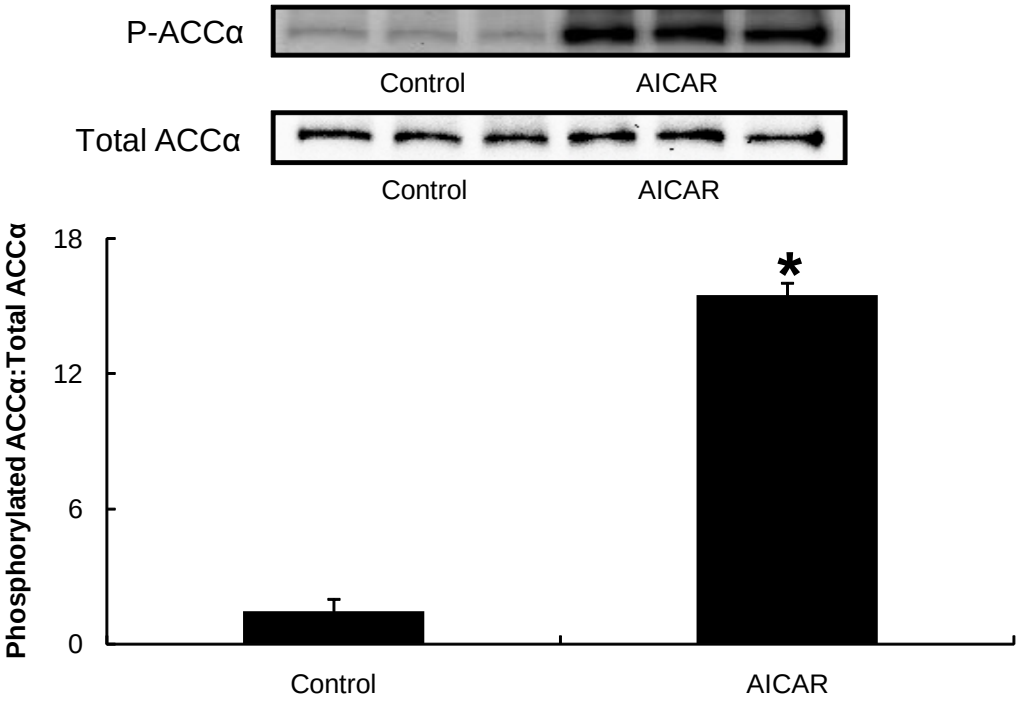


Figure 5.4. Effect of treating bovine mammary epithelial cells (MAC-T) with or without AICAR (600 μM) for 1 h on acetyl-CoA carboxylase (ACC) activity. Error bars represent SEM for two independent experiments. An asterisk indicates a significant difference from control, $P < 0.01$.

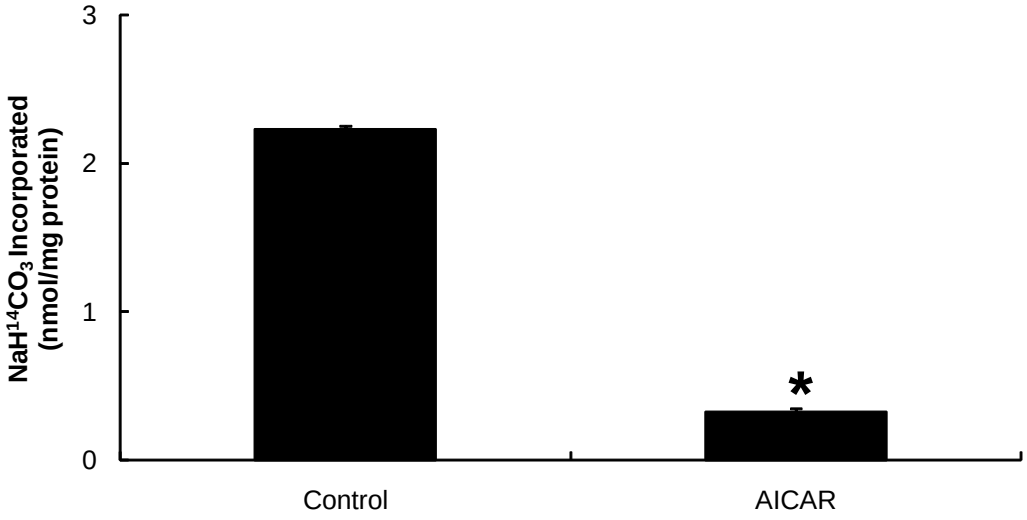


Figure 5.5. Effect of treating bovine mammary epithelial cells (MAC-T) with or without AICAR (600 μM) for 24 h on lipogenic gene expression. (A) Lipogenic genes evaluated were acetyl-CoA carboxylase-α (ACCα), fatty acid synthase (FAS), lipoprotein lipase (LPL), and fatty acid binding protein-3 (FABP3). (B) Genes involved in the synthesis of triacylglycerol were also evaluated. These included glycerol phosphate acyltransferase (GPAT) and acylglycerol phosphate acyltransferase (AGPAT). Solid and open bars represent control and AICAR treatments, respectively. Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. An asterisk indicates a significant difference in ΔCt from control, $P < 0.01$.

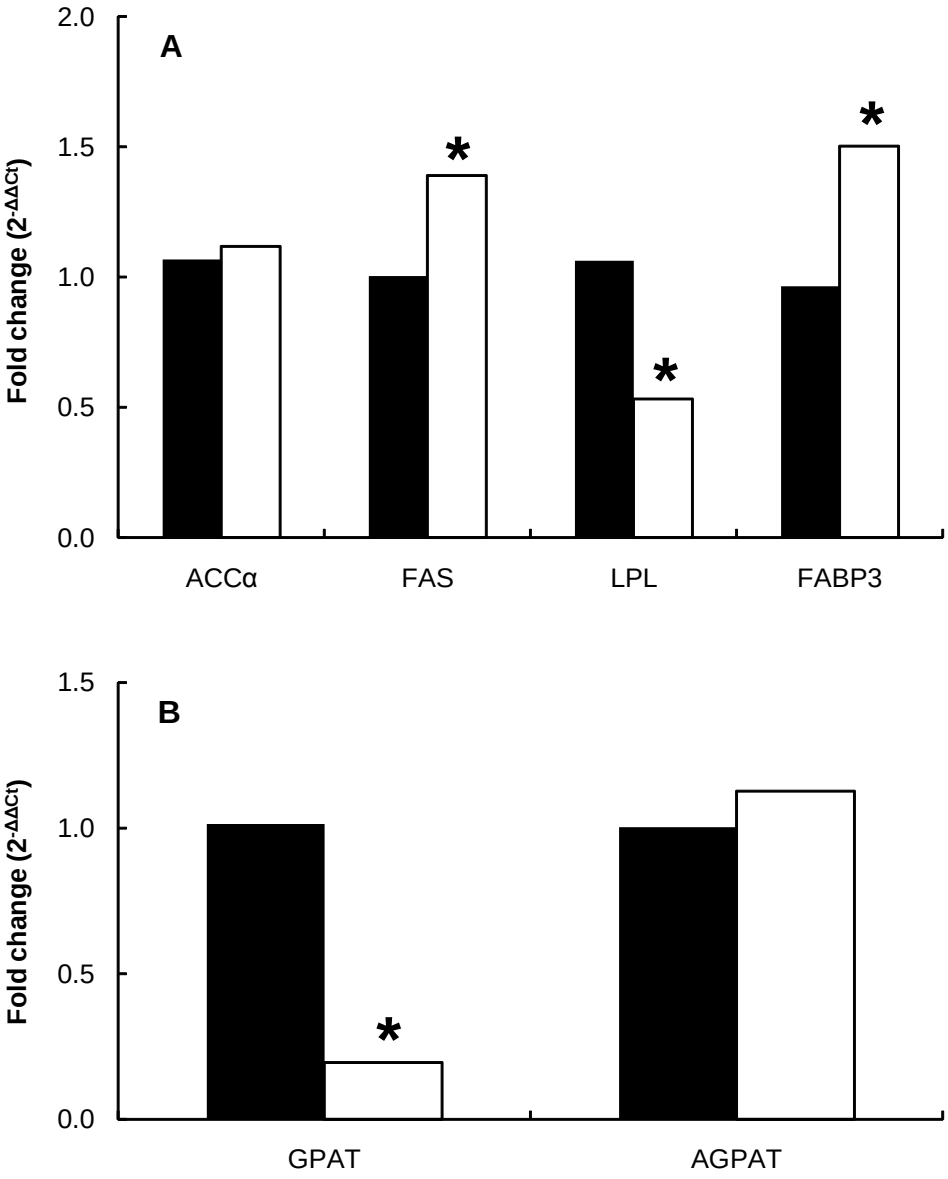
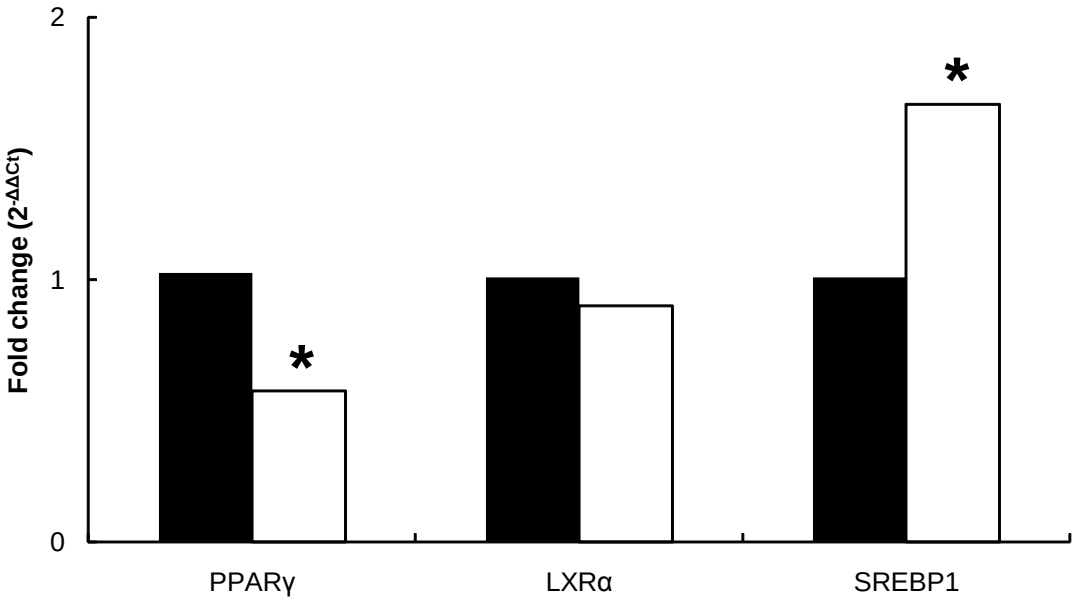


Figure 5.6. Effect of treating bovine mammary epithelial cells (MAC-T) with or without AICAR (600 μM) for 24 h on various lipogenic transcription factor gene expression. Transcription factors evaluated were peroxisome proliferator-activated receptor-γ (PPARγ), liver X receptor-α (LXRα), and sterol regulatory element binding protein-1 (SREBP1). Solid and open bars represent control and AICAR treatments, respectively. Data is illustrated as least squares means and represents two independent experiments with three replicates per experiment. An asterisk indicates a significant difference in ΔCt from control, $P < 0.01$.



glucose in the culture medium for 4 h significantly increased the ratio of P-ACCα to total ACCα (Figure 5.7).

Ca^{2+}/calmodulin-dependent kinase kinase is an AMPK kinase sensitive to rises in intracellular Ca^{2+}. Ionomycin, a Ca^{2+} ionophore, is often used to study CaMKK because it increases intracellular Ca^{2+}. Increases in intracellular Ca^{2+} result in the phosphorylation of AMPK (Hurley et al., 2005; Tamas et al., 2006). In the present study, treatment of MAC-T with ionomycin for 1 h resulted in a significant inactivation of ACCα (Figure 5.8).

DISCUSSION

Activation of AMPK occurs when AMP binds to Bateman domains on its γ subunit resulting in a conformational change permitting the phosphorylation of the α subunit. The β subunit of AMPK structurally supports the α and γ subunits of AMPK. The mRNA of all three subunits of AMPK were found in bovine adipose, liver, muscle, and mammary tissue in addition to MAC-T. The relative expression of α, β, and γ AMPK was greatest in muscle compared to other tissues and MAC-T. Likewise, AMPK mRNA is most abundant in skeletal muscle compared to other tissues (Verhoeven et al., 1995; Woods et al., 1996). We also observed the absence of the γ3 isoform in mammary tissue and MAC-T. Mahlapuu and coworkers (2004) determined that the γ3 isoform of AMPK is predominantly expressed in skeletal muscle, whereas the γ1 and γ2 isoforms show broad tissue distributions (Cheung et al., 2000; Buhl et al., 2001). Lastly, we also failed to detect the α2 isoform in MAC-T. In rats, the mRNA expression of α2 AMPK is

Figure 5.7. Effect of incubating bovine mammary epithelial cells (MAC-T) with Dulbecco's Modified Eagle's Medium (DMEM) with or without glucose for 4 h on ratio of phosphorylated-ACCα (P-ACCα) to total ACCα protein abundance. Error bars represent SEM for two independent experiments with three replicates per experiment. An asterisk indicates a significant difference from control, $P < 0.0001$.

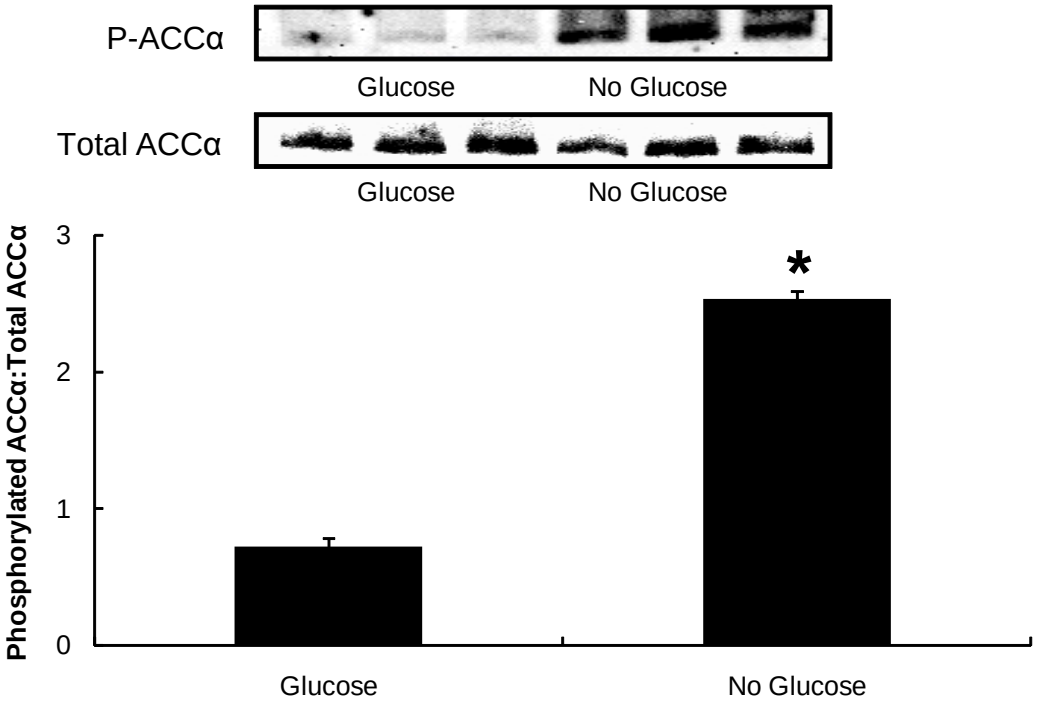
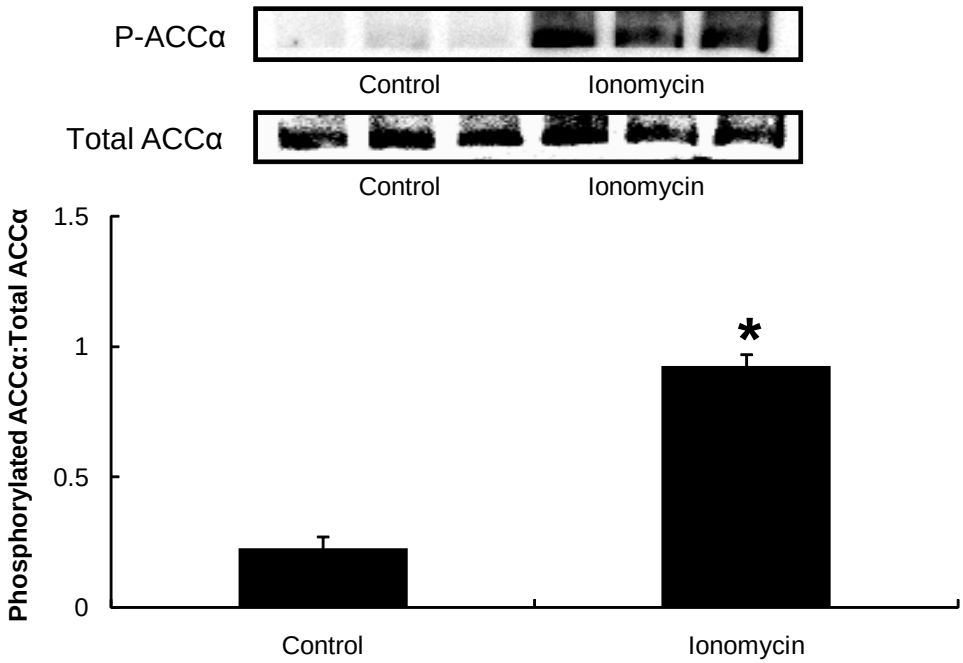


Figure 5.8. Effect of treating bovine mammary epithelial cells (MAC-T) with either control (DMSO) or ionomycin (1 μM) for 1 h on ratio of phosphorylated-ACCα (P-ACCα) to total ACCα protein abundance. Error bars represent SEM for two independent experiments with three replicates per experiment. An asterisk indicates a significant difference from control, $P < 0.0001$.



greatest in skeletal muscle and liver while α1 AMPK is expressed ubiquitously (Stapleton et al., 1996; Stephens et al., 2002). The phosphorylation of both α isoforms by upstream AMPK kinases has been well documented (Hawley et al., 2003; Hawley et al., 2005); therefore, use of MAC-T as an *in vitro* model of the mammary gland is appropriate.

Activation of AMPK can regulate lipid synthesis (see review by Hardie and Pan (2002)). In the present study, acute AICAR (200-600 µM) treatment significantly decreased *de novo* fatty acid synthesis in MAC-T up to 85%. Similar observations were observed with chronic AICAR (400-600 µM) treatment. However, treatment with 200 µM AICAR for 24 h significantly increased fatty acid synthesis as measured by radiolabeled acetate incorporation into lipid and this was unexpected. Administration of AICAR decreased lipid synthesis in human hepatoma cells (Brusq et al., 2006). In certain cell types and species, AMPK can regulate the transcription of various lipogenic genes (Zhou et al., 2001; Diraison et al., 2004). Treatment of MAC-T with 600 µM AICAR for 24 h resulted in 38% and 66% increases in the mRNA expression of FAS and SREBP1, respectively. Sterol regulatory element binding protein-1 can regulate the transcription of FAS (Magana and Osborne, 1996). It is possible that FAS expression was upregulated by SREBP1 as a compensatory mechanism in response to the dramatic reduction in lipid synthesis. This finding may also explain why we observed a significant increase in acetate incorporation after chronic treatment with 200 µM AICAR. Whether chronic treatment of MAC-T with 200 µM AICAR increases FAS and SREBP1 expression remains to be verified.

Activation of AMPK by AICAR results in the phosphorylation of ACC and its inactivation (Zhou et al., 2001; Park et al., 2002b). In the present study, treating MAC-T with 600 µM AICAR significantly increased the ratio of P-ACCα to total ACCα by 959%. In addition, the activity of ACC decreased by 86%. Increasing concentrations of AICAR linearly increases ZMP production in muscle of rats (Merrill et al., 1997). 5-Aminoimidazole-4-carboxamide ribonucleoside monophosphate is an AMP analog which can bind to the γ subunit of AMPK promoting phosphorylation. The mRNA expression of the α2 isoform of AMPK was absent in MAC-T. Therefore, AICAR was converted to ZMP subsequently increasing the phosphorylation of α1 AMPK and resulting in the inactivation of ACCα in MAC-T. The dramatic increase in the ratio of P-ACCα to total ACCα most likely contributed to the significant decrease in acetate incorporation in response to acute and chronic treatment of AICAR.

Lipoprotein lipase is essential for the hydrolysis of TAG and subsequent uptake of preformed fatty acids. The mRNA expression of LPL decreased by 50% in response to AMPK activation. Knockdown of AMPKα in 3T3-L1 adipocytes results in increased activation of LPL (Kim et al., 2007). In addition, stimulation of LPL activity is likely an AMPK-dependent process in cardiac muscle (An et al., 2005). Interestingly, we also observed a 44% decrease in the expression of PPARγ. A PPARy response element has been identified within the promoter region of LPL (Schoonjans et al., 1996a). In addition, AICAR can dramatically decrease PPARγ protein abundance in 3T3-L1 adipocytes (Habinowski and Witters, 2001). We propose that the dramatic reduction in *de novo* fatty acid synthesis decreases the demand for the incorporation of preformed fatty acids into TAG and therefore decreased the expression of LPL by decreasing the

expression of PPARγ. In support, activation of AMPK in adipocytes dramatically suppresses fatty acid uptake (Gaidhu et al., 2009). Gaidhu and coworkers (2009) propose that fatty acid uptake is decreased in order to prevent the esterification of excess fatty acids, an energy consuming process.

The mRNA expression of GPAT was decreased by 81% in response to AICAR; however, AGPAT mRNA expression was not modified by treatment. Glycerol-3-phosphate acyltransferase is responsible for the first acylation in TAG synthesis. The incorporation of radiolabeled oleate into TAG is decreased in response to AICAR (Muoio et al., 1999). Muoio and coworkers (1999) also observed a significant decrease in GPAT activity. We believe GPAT expression was reduced in response to a dramatic reduction in substrate (short-, medium-, and long-chain fatty acids) caused by the inactivation of ACCα and the downregulation of LPL mRNA expression.

Fatty acid binding protein-3 is closely associated with the β-oxidation of fatty acids (Hertzel and Bernlohr, 2000). In addition, AMPK activation has been shown to increase FABP3 mRNA expression (Lee et al., 2006). In the present study, the mRNA expression of FABP3 increased 56%. We believe the intracellular concentration of preformed, long-chain fatty acids was increased due to a decrease in TAG synthesis caused by a reduction in *de novo* fatty acid synthesis. Therefore, the increase in FABP3 may have supported an increase in mitochondrial β-oxidation of fatty acids.

AMP-activated protein kinase is sensitive to decreases in energy supply. The bovine mammary epithelial cell utilizes acetate as the primary source of energy supply. However, glucose can also be oxidized in the bovine mammary epithelial cell in addition to serving as a substrate for lactose synthesis. Removal of glucose from the incubation

medium for 4 h resulted in a 251% increase in the P-ACCα to total ACCα ratio (Figure 5.7). Removal of glucose from the cell culture medium activates AMPK in cardiac myocytes (An et al., 2005). Low glucose supply also increases the AMP/ATP and ADP/ATP ratios in pancreatic β cells resulting in the activation of AMPK (Salt et al., 1998). We propose that removal of energy supply can result in the phosphorylation of ACCα by AMPK thereby inhibiting fatty acid synthesis, an energy consuming process in the bovine mammary epithelial cell.

Ca^{2+}/calmodulin-dependent kinase kinase is one of two known upstream kinases of AMPK. In the present study, 1 μM ionomycin significantly increased the ratio of P-ACCα to total ACCα by 308%. Ionomycin increased the phosphorylation of AMPK and its activity by ~500% in HeLa cells (Hurley et al., 2005). STO-609, a CaMKK inhibitor, completely reversed the effects of ionomycin on AMPK phosphorylation. In addition, ionomycin-stimulated AMPK activity, AMPK phosphorylation, and ACC phosphorylation are substantially reduced in HeLa cells transfected with small interfering RNAs specific for both isoforms of CaMKK (Hurley et al., 2005). Hurley and coworkers (2005) also observed that the activation of AMPK by ionomycin is not impaired in LKB1 knockout murine embryo fibroblasts. We can therefore hypothesize that ionomycin inactivated ACCα in a CaMKK-dependent, LKB1-independent manner. Further research is needed to indentify the presence of CaMKK in MAC-T and its ability to regulate lipid synthesis.

In conclusion, we identified the presence of AMPK mRNA in various bovine tissues and MAC-T. In MAC-T, we conclude that AMPK regulates the synthesis and utilization of fatty acids by phosphorylating ACCα and modifying gene transcription. We also propose that AMPK is able to inhibit fatty acid synthesis, an energy consuming

process, in response to decreases in energy supply. In addition, our results suggest the presence of CaMKK and its potential regulation of lipid synthesis in MAC-T.

CHAPTER 6

Conclusions

The synthesis of fatty acids in bovine mammary epithelial cells is controlled by various regulatory proteins which include SREBP1, LXR, and AMPK. We conclude that *de novo* fatty acid synthesis is inhibited by polyunsaturated fatty acids by downregulating the expression of SREBP1 in BME-UV bovine mammary epithelial cells. In BME-UV, we demonstrate that LXR activation enhances *de novo* fatty acid synthesis by upregulating the expression of SREBP1. Thus, we conclude that SREBP1 is a LXR target and hypothesize that activation of LXR in the mammary gland may modify milk fat production. However, LXR activation was unable to prevent observed decreases in *de novo* fatty acid synthesis in response to polyunsaturated fatty acids. *In vivo*, LXR activation may not be able to prevent the inhibitory effect of *trans*-10, *cis*-12 CLA; therefore, LXR activation wouldn't be an effective strategy when managing milk fat depression. Activation of AMPK dramatically decreased *de novo* fatty acid synthesis in MAC-T bovine mammary epithelial cells by inactivating ACCα. In addition to inactivating ACCα, we also demonstrate that AMPK activation can modify gene transcription. Identifying AMPK as a molecular target capable of modifying energy substrate utilization may result in the development of new technologies that increase milk production or modify milk composition during periods of increased energy demand. We also provide evidence that LKB1 and CaMKK are expressed in MAC-T. With additional research, these AMPK kinases may prove to be additional targets for modifying milk fat production in dairy cows.

Milk fat is not only a major component of the economic value of milk, but represents a significant portion of the energy cost associated with lactation. It may be advantageous for producers to induce milk fat depression during periods of limited feedstuff availability (i.e. pasture feeding during drought) or during the periparturient period. A reduction in milk fat yield can allow for a repartitioning of nutrients to support increased milk and milk protein yield. For example, milk protein synthesis in grazing dairy cows is often limited by energy availability. A decrease in milk fat production could reduce the energy requirements for milk production and as a consequence dietary CLA could increase milk protein yield. In addition to performance, reductions in milk fat yield may improve animal health. For example, early lactation is often associated with a period of negative energy balance which is known to negatively affect reproduction and increase the susceptibility of dairy cows to various metabolic disorders, thereby limiting milk yield throughout lactation. Reducing milk fat production during negative energy balance may alleviate the severity of increased energy demand and improve animal performance and well-being. Better understanding the molecular regulation milk fat synthesis will give producers the ability to not only regulate milk fat synthesis but also control nutrient utilization.

Including the inhibitory effects of AMPK activation on milk fat synthesis, AMPK may also inhibit the synthesis of other milk components including milk protein and decrease milk production by regulating the uptake of glucose in the lactating mammary gland. Since glucose is the precursor of milk lactose which is the major osmotic component of milk, a decrease in glucose availability could result in a decrease in milk synthesis. Therefore, activation of AMPK may decrease the production of milk lactose

which could decrease milk production. To avoid this loss in potential profit, the American dairy producer should strive to ensure their dairy cows are consuming enough dietary energy to meet the dairy cow's demands of maintenance, growth, reproduction, and lactation. If producers fail to meet this standard, AMPK will most likely become activated in the lactating mammary gland resulting in a decrease in milk components and yield.

Based on our findings, we feel that LXR and AMPK play an active role in the regulation of *de novo* fatty acid synthesis in bovine mammary epithelial cells. Therefore, the development of technologies that control LXR or AMPK activation may provide dairy producers with additional management strategies to modify milk fat production and composition to meet consumer demand and maximize profitability. Future research should identify the relative contribution of SREBP1 and LXR to the *de novo* synthesis of fatty acids in the bovine mammary gland. Also, identifying endogenous LXR ligands should be determined to further elaborate the role of LXR in the mammary gland. In the bovine mammary gland, better understanding the role of AMPK activation in response to energy demand is needed. Characterizing the effect of AMPK activation on other metabolic pathways such as fatty acid uptake and oxidation should also be investigated. Lastly, identifying the presence of LKB1 and CaMKK and their relative contributions to the activation of AMPK in the bovine mammary gland is necessary.

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