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FATTY ACIDS AND LIPOGENESIS IN RUMINANT ADIPOCYTES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Animal and Veterinary Sciences

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by
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ABSTRACT

Obsesity, the excess deposition of white adipose tissue, is a growing problem in the U.S. and other developed countries. Formerly thought to be inert, adipose tissue is now recognized as a dynamic endocrine organ with its secretion of adipokines and a newly proposed class hormone class "lipokine". Adipocytes are the functional unit of adipose tissue and can influence the tissue through hyperplasic and hypertrophic growth. In order to investigate the mechanisms involved in adipogenesis and lipogenesis of adipose tissue, stromal vascular cultures were isolated from adipose tissue of finishing cattle for use in experiments.

There is a positive relationship between lipogenic gene expression and increased energy in steer diets. The objectives of the studies presented in Chapter 2 were to determine if differences in fatty acid profiles or gene expression exist when adipocytes are exposed to different, simulated energy sources: linoleic acid, insulin, or both following differentiation. With limited information in the literature about the timing of lipid uptake and fatty acid composition in differentiating bovine adipocytes, a secondary objective of these studies was to evaluate fatty acid composition over time. Overall, results from these studies indicate that fatty acid composition changes over time post-differentiation and is modulated by linoleic acid supplementation. Expression of SCD1 mRNA was up-regulated prior to changes in fatty acid desaturation profiles. In addition, linoleic acid treatment was incorporated into cells and its supplementation decreased production of *de novo* fatty acids synthesis or increased β-oxidation of fatty acids or both.

Recent evidence links palmitoleic acid to decreased lipogenesis in murine hepatocytes. Since adipocytes are the primary site of lipogenesis in ruminants, the objectives of the experiment in Chapter 3 was to determine if a similar effect could be seen in bovine adipocytes and titrate an effective dose of palmitoleic acid supplementation. Concurrent with palmitoleic acid, *cis*-vaccenic (C18:1 *cis*-11) and eicosenoic (C20:1 *cis*-13) acids increased linearly with palmitoleic acid supplementation in bovine adipocytes. In addition, activity and mRNA expression of several lipogenic genes were down-regulated and β-oxidation was increased in response to palmitoleic acid supplementation. Therefore, palmitoleic acid was having an anti-lipogenic effect on the bovine adipocyte cultures.

The elevated presence of suspected elongation products of palmtioleic acid, *cis*-vaccenic and eicosenoic acids, in cultures supplemented with palmitoleic acid left doubt as to which fatty acid contributes to anti-lipogenic effects. The objectives of the studies performed in Chapter 4 were to confirm *cis*-vaccenic and eicosenoic acids as elongation products of palmtioleic acid using a stable isotope tracer and test lipogenic effects of *cis*-vaccenic acid. *Cis*-vaccenic and eicosenoic acids are, indeed, direct elongation products of palmtioleic acid. In addition, *cis*-vaccenic acid decreased lipogenesis rates, but did not affect desaturation. Therefore, palmitoleic acid differentially affects aspects of lipogenesis relative to its elongation products. Overall, the results of this work advance our understanding of the biological mechanisms underlying lipogenesis.

DEDICATION

I would like to dedicate this work to my loving and supportive husband.

ACKNOWLEDGEMENTS

Thank you, Dr. Duckett, for making this all possible. I have sincerely enjoyed my time working with you at Clemson University. It has been a wonderful experience that I intend to share with others. You always had my best interests at heart and pushed me to excel. I could not have asked for a better mentor. Also, 'thank you' to my committee members, Drs. Karen Burg, Tom Jenkins, Scott Pratt, and Steve Ellis (unofficial member) for their various and invaluable contributions to this work and to my professional development.

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CHAPTER 1: FATTY ACIDS IN RUMINANTS

INTRODUCTION

Nutrition plays an integral role in animal production systems; it impacts maintenance, growth, health, and reproduction. Maximizing efficiency and optimizing nutrient partitioning of the diet are economical goals of most producers. An animal's excess energy is stored as fat in adipocytes, which are the functional, energy-storage units of the body. Fat has both positive and negative aspects for producers. In the beef industry, USDA grading standards estimate cutability and eating quality of the lean tissue to determine carcass value. Yield and quality grades applied to beef carcasses are highly influenced by the abundance of subcutaneous and intramuscular fat, respectively. Excess subcutaneous fat can decrease carcass value in addition to wasting dietary energy, an estimated financial loss of $1.3 billion annually (National Beef Quality Audit, 2000). However, intramuscular fat, or marbling, is an estimate of eating quality of the meat and usually results in a premium price. Tenderness, the key determinant of a steak's eating quality, is associated with marbling. In addition, consumers prefer steaks that are high in marbling rating them better in terms of juiciness, flavor, and overall acceptability than steaks of the same tenderness level with low marbling scores (Killinger et al., 2004). Therefore, the abundance of fat in meat products has financial and health implications to both producers and consumers.

The composition of fat in animal products is also important to consumers and producers. Meat and milk are the primary end-products of economic importance in animal-production agriculture. Flavor, a major indicator of consumer acceptability and

species-determination, can be greatly affected by fatty acid composition. Health concerns have arisen in recent years regarding fatty acid composition of animal products in the human diet. In general, saturated fatty acids (SFA) in the human diet are associated with elevated cholesterol levels (Hegsted et al., 1965), an exception being stearic acid (C18:0) (Denke and Grundy, 1991). However, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the human diet can reduce serum cholesterol levels (Mattson and Grundy, 1985; Table 1). The composition and quantity of white adipose tissue found in meat is greatly influenced by the animal's nutrition. For instance, cattle finished on grain have greater MUFA in intramuscular fat compared with cattle finished on forage, which have greater C18:0 and PUFA (Williams et al., 1983). The time on feed can also impact quantity and composition of intramuscular fat in cattle (Duckett et al., 1993). During the finishing-stage in cattle production, approximately 14 to 18 mo. of age, intramuscular fat deposition is the result of both hyperplasia, increase in cell number, and hypertrophy, increase in cell size (Hood and Allen, 1973). Although cell number and size both increase during animal maturation, 70% of fat accumulation is the result of hypertrophy (Robelin et al., 1985) with most hyperplasia of subcutaneous and perirenal adipose depots complete by 8 mo. of age (Hood and Allen, 1973). Preadipocytes, cells responsible for hyperplasia, are of mesenchymal lineage and have the ability to propagate or commit to differentiation (Gesta et al., 2007). With an increase in excess energy, preadipocytes differentiate into mature adipocytes, which have the ability to synthesize, take up, and store fat. Adopting feeding, targeted molecular

biotechnology strategies, or both to alter abundance and composition of adipose tissue could improve production efficiency and quality of meat.

Prior to the discovery of leptin (Zhang et al., 1994), the primary effector of satiety, white adipose tissue was thought to be solely an energy-storage unit, inert, with no metabolic function as reviewed by McGillis (2005). Since then, adipokines, such as adiponectin, have been discovered as cell-to-cell signaling hormones which are exclusively expressed by adipocytes and associated with several inflammatory responses (MacDougald and Burant, 2007). Additionally, eicosanoids are a class of hormones derived from essential fatty acids, predominantly arachidonic acid (C20:4n-6). Three main types of eicosanoids, leukotrienes, thromboxanes, and prostaglandins, have also been associated with reproduction, inflammatory, and other immune-based physiologic processes. An even newer concept in the role for adipocytes in whole body metabolism is that fatty acids, themselves, can act as cell signals. Cao et al. (2008) identified a particular fatty acid, palmitoleic acid (C16:1 *cis*-9), present in low levels throughout bodily tissues that acts as a hormone to regulate lipogenesis and coined the term "lipokine". While a full understanding of the regulatory functions of adipocytes and their constituents has not been realized in the literature, data support adipose tissue to be a diverse endocrine organ that can affect whole body metabolism in addition to serving as an energy reservoir. Many disease states are already associated with altered fatty acid metabolism including cardiovascular disease, type II diabetes, obesity, and some cancers. Less known are the regulatory functions of adipocytes that maintain homeostasis or promote health.

FATTY ACIDS AND LIPOGENESIS

Fatty acids are generally classified as saturated or unsaturated, based on the bonding pattern that occurs within the hydrocarbon skeleton of the fatty acid molecule. Fatty acids are composed of a carboxylic acid group attached to a hydrocarbon chain of varying length. If each carbon in the chain has the maximum possible number of hydrogen atoms attached to it, or no double bonds, the fatty acid molecule is considered saturated. Conversely, if there is at least one double bond between two carbon atoms in the hydrocarbon chain, the fatty acid is unsaturated. A MUFA has only one double bond and a PUFA has two or more double bonds present within its hydrocarbon chain. Most animal fats are high in SFA, whereas plant oils are mostly comprised of PUFA. The two essential PUFA required by ruminants are linoleic acid (C18:2n-6), an omega-6 fatty acid, and linolenic acid (C18:3n-3), an omega-3 fatty acid. With the exception of common names, fatty acid nomenclature uses the carbon length, double bond location, and geometric structure to designate specific fatty acids. 'Lipid numbers' is the most widely used naming system. It uses a Cx:yz structure, where x is the number of carbons, y is the number of double bonds, and *z* lists the geometry and location of each double bond as *cis*- or *trans*-, counting from the carboxylic acid end. For example, oleic acid (C18:1 *cis*-9) is an 18 carbon fatty acid with one double bond in the *cis*- position between the 9^{th} and 10^{th} carbons. With some PUFA, in lieu of listing all double bond locations and geometry, an omega designation is often used. As the name implies, omega refers to the last carbon in the hydrocarbon chain, or the methyl carbon. Counting back toward the carboxylic acid end, the location of the first double bond is used for naming. For

instance, linolenic acid can be listed as C18:3 *cis*-9, *cis*-12, *cis*-15 or simply C18:3n-3 because the double bond is on the third carbon away from the methyl end.

Fatty acids stored in adipocytes can come from two sources: diet or *de novo* synthesis. For most animals, dietary fatty acids are separated from their larger fat particles and lipid molecules in the lumen of the small intestine by bile salts and pancreatic lipase. Fatty acids are absorbed into intestinal epithelium, reassembled into triglycerides, and passed into the bloodstream for transport to the liver before traveling to the rest of the body as components of lipoproteins. The liver is a critical organ in the conversion of fatty acids into various forms which are used throughout the body. Dietary fatty acids are then incorporated into body tissues and fluids where they can play a dynamic role in hormone synthesis. Due to biohydrogenation of lipids in rumen of pregastric fermentors, energy metabolism has developed a little differently in these animals. Ruminants have adapted over thousands of years to digest a wide variety of feedstuffs with the aid of a symbiotic relationship with microbial organisms in their gut. These microbes are capable of digesting structural carbohydrates that most mammals are incapable of utilizing. However, due to the microbial rumen environment, most dietary nutrients are only available to the animal second-hand. The primary energy substrate ruminants receive from the diet is not glucose, but volatile fatty acids (VFA), which are waste products of carbohydrate fermentation by microbes (Vernon, 1980; Smith and Crouse, 1984). Lipids are another example of a microbially-modified nutrient, as dietary unsaturated fatty acids undergo biohydrogenation in the rumen.

Unsaturated fatty acids are toxic to the rumen's microbial population, especially to those responsible for fiber digestion; therefore, the microbes have adapted the use of biohydrogenation to solve this problem. Biohydrogenation sequentially removes double bonds and makes unsaturated fatty acids increasingly more saturated. Biohydrogenation decreases, but does not eliminate, the amount of PUFA available to the animal. In forage-based diets, PUFA are in the form of galactolipids. This is a molecule that has two fatty acids attached to a glycerol backbone with a galactose bound to the third carbon (monogalactosyl diacylglycerol). Additional galactose residues can be attached to the glycerol-bound galactose, increasing the fermentability of the galactolipid. In grain-based diets, triglycerides are the primary form of lipid. A triglyceride is a molecule with three fatty acids bound to a glycerol backbone. For these lipids to be absorbed, they are broken down into free fatty acids (FFA), galactose, and glycerol. Microbes produce galactosidases which cleave galactoses from the glycerol molecule, resulting in free sugars and diacylglycerol. Microbial lipases cleave the fatty acids from the glycerol. Also, microbes in the rumen have general esterases and extracellular lipase that result in the separation of triglycerides and diacylglycerides into FFA and glycerol. Once the fatty acids are 'free', they are susceptible to biohydrogenation. There are several pathways for biohydrogenation, and research has not clearly elucidated all the possible routes of biohydrogenation. The process by which double bonds are removed from the carbon backbone are PUFA specific and have evolved to use the least amount of energy based on the positioning of the bonds. The primary end-product of C18:2n-6 and C18:3n-3 biohydrogenation is C18:0 (Noble et al., 1974; Jenkins and Bridges, 2007; Lee and

Jenkins, 2011). However, not all fatty acid molecules are fully saturated during the biohydrogenation process; PUFA and biohydrogenation intermediates escape the rumen and can be found in animal plasma, tissue, and milk.

Microbes in the rumen ferment carbohydrates into VFA which are absorbed across the epithelial lining of the rumen, reticulum, and omasum. There is no active transport of VFA; they travel by passive diffusion down the concentration gradient. The rate of absorption, equal for all pregastric stomach compartments, is positively correlated with decreasing pH and increasing VFA chain length. The three predominant VFA are acetate, propionate, and butyrate, and each has a distinct fate in metabolism. A small amount of propionate, about 5%, is converted to lactate by the rumen epithelium and the majority is sent to the liver for gluconeogenesis. Butyrate is extensively metabolized into ketone bodies in the rumen epithelium: acetoactetate, β-hydroxybutyrate, or acetone. Approximately 5% of acetate is converted into ketone bodies by the rumen epithelial cells, but the majority of acetate is transported to the liver where it is converted to acetyl-CoA. Of this acetyl-CoA, 80% is circulated to the rest of the body while the remaining 20% enters the citric acid cycle for ATP production. In the peripheral tissues, acetyl-CoA is predominantly used as a substrate for *de novo* fatty acid synthesis in adipose tissue (Figure 1). Cattle have very little to no citrate lyase activity. Therefore, they use very little glucose as a carbon source for fatty acid synthesis, but can utilize acetyl-CoA directly. However, glucose contributes a relatively greater proportion of acetyl units to fatty acid biosynthesis in intramuscular adipose tissue compared with subcutaneous adipose tissue in cattle (Smith and Crouse, 1984). In ruminants, the primary site of fatty

acid synthesis is white adipose tissue, as opposed to most nonruminants where the primary site of fatty acid synthesis is the liver.

Fatty acid synthesis begins with the committed step of adding a carboxyl group to acetyl-CoA, forming malonyl-CoA, by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FASN) is a large, multimeric protein that binds acetate and malonyl-CoA or another fatty acyl-CoA. It uses NADPH in a series of sequential reactions to condense the substrates into a SFA product that is two carbons longer than the original malonyl- or fatty acyl-CoA. The process is repeated until the fatty acid reaches a length of 16 carbons, palmitoyl-CoA (Figure 1). Short and medium chain fatty acids can enter this process at any step. As FASN is not able to elongate fatty acids greater than 16C, a family of elongase enzymes (ELOVL) is responsible for elongating fatty acids after this point (Figure 1). Stearoyl-CoA desaturase (SCD) is a Δ^9 desaturase that it inserts a *cis*-double bond between carbons 9 and 10 on the hydrocarbon chain, counting from the carboxyl end (Ntambi, 1999). Animals do not have a Δ^{12} or Δ^{15} desaturase; therefore, dietary plant material is the primary source C18:2 n-6 and C18:3 n-3. Arachidonic acid (C20:4 n-6) is also considered an essential fatty acid in most mammalian species due to a reduced capacity or inability to synthesize it from C18:2 n-6 in sufficient quantities to meet the demands of the body. These pathways in combination with dietary fatty acids subjected to biohydrogenation result in a variety of fatty acid products. In order from greatest to least, the major fatty acids present in beef are C18:1 *cis*-9, palmitic acid (C16:0), C18:0, and C18:2 n-6 (Duckett et al., 1993). The order of predominance changes if cattle are finished on a forage-based diet compared with the traditional grain-

based diet to: C18:1 *cis*-9, C16:0, C18:0, C16:1, and C18:2 n-6 (Pavan and Duckett, 2008). Other fatty acids of importance to human health that are present in beef are conjugated linoleic acids (CLA) and omega-3 fatty acids (Table 1). Once synthesized or processed in adipocytes, free fatty acids can be packaged into triglycerides for storage, sphingolipids and phospholipids for membrane components, cholesterols and eicosanoids for hormone production. In addition, fatty acids can undergo β-oxidation to provide ATP as energy to cells and tissues (Figure 1).

ADIPOGENESIS

Adipogenesis is defined as the differentiation of preadipocytes into functional adipocytes. Cellular changes due to adipogenesis are characterized by cell morphology change, gene and protein expression, and function of the cell. Differentiation is initiated through extracellular signals altering gene expression at the transcriptional and translation level. The full developmental origins of preadipose tissue from a single cell are unknown, but are thought to have mesodermal origins (Ntambi and Kim, 2000; Cornelius et al., 1994). In humans, preadipocytes begin to differentiate into adipose tissue during late embryonic development and majority of differentiation occurring shortly after birth (Burdi et al., 1985). In mice and rats, preadipocytes do not begin to differentiate until after birth (Ailhaud et al., 1992). Early-onset obesity in humans is a result of both hyperplasia and hypertrophy of adipose tissue, but adult-onset obesity is primarily a result of adipocyte hypertrophy (Hirsch and Batchelor, 1976). However, morbidly obese humans, those greater than 170% of ideal body fat, display both hyperplasia and hypertrophy of adipocytes (Hirsch and Batchelor, 1976). Fat deposition in finishing cattle is resultant from a combination hyperplasia and hypertrophy; 70% of which is due to the latter (Robelin et al., 1985).

Most of the work characterizing adipogenesis has been conducted using murine *in vivo* and *in vitro* models. In cell culture models, a hormone cocktail is commonly used to initiate differentiation, although fatty acids or sheer confluence of the cells can also induce a level of differentiation (Ntambi and Kim, 2000). *In vivo*, the process is less clear, but assumed to be regulated through energy status signaling. With increasing

excess energy, differentiation of cells increases to meet the storage demands (Ntambi and Kim, 2000). The changes in gene transcription that ultimately lead to altered cell function as mature adipocytes are initiated by transcription factors (Figure 2). Some of the first transcription factors to increase at the onset of differentiation are CCAAT/enhancer binding proteins (C/EBP)-α, –β, and –δ. The C/EBP-α isoform is thought to be mediated by C/EBP-β and –δ (Ntambi and Kim, 2000). These C/EBP increase within 1 h and dissipate within 24 h in differentiating cells (Cornelius et al., 1994). Next, peroxisome proliferator-activated receptor (PPAR)-α and –γ increase and are also thought to be mediated by C/EBP-β and –δ (Clarke et al., 1997). Based on mouse research, the master regulator of adipocyte regulation is classically stated as PPARγ. It homodimerizes or heterodimerizes with retinoid X receptor or retinoic acid receptor to promote transcription of lipogenic genes (Rosen et al., 2000). Adipocyte protein 2 [aP2, also known as fatty acid binding protein 4 (FABP4)], sterol regulatory element binding protein (SREBP), and SCD mRNA expression increase around day 2 to 4 after initial differentiation (Amri et al., 1991). Sterol regulatory element binding protein is proteolytically matured by SREBP-cleavage activating protein which senses falling sterol levels within the cell and transports SREBP from the membrane of the endoplasmic reticulum to the Golgi complex. In the Golgi membrane, site 1 protease and site 2 protease sequentially cleave SREBP into its mature form, n-SREBP, which is transported to the nucleus of the cell. There, it binds to the sterol response element of lipogenic genes. The three known isoforms of SREBP are -1a, -1c, and -2. All SREBP-responsive genes can be induced by SREBP-1a, but SREBP-1c and SREBP-2 specifically

activate fatty acid and cholesterol synthesis genes, respectively (Brown and Goldstein, 1997; Horton et al., 2002; Eberlé et al., 2004).

Stearoyl-CoA desaturase could be used as an indicator of differentiation timing *in vivo* (Martin et al., 1999). Growing cattle, 5 to 12 mo of age, showed increased SCD gene expression in subcutaneous adipose tissue that peaked at 12 mo of age (Martin et al., 1999). Following this peak, cellularity of the adipose tissue changed with increased lipid filling. As stated earlier, cattle finished on high concentrate diets have altered fatty acid profiles and lipid deposition rates compared to pasture-finished animals (Pavan and Duckett, 2008). Therefore, diets of finishing cattle could be used to impact adipose tissue at the level of differentiating cells, lipid-filling adipocytes, or both. In addition to diet and depot having an effect on adipogenesis *in vivo*, the depot of fat that preadipocytes are cultured from can have a profound effect on their performance *in vitro*. For instance, Grant et al. (2008a) reported that cells isolated from subcutaneous fat had 6.4-fold greater percentage of differentiated cells within individual colonies than cells isolated from intramuscular fat. In addition, cultures derived from subcutaneous fat had greater response to differentiation media than cultures from omental adipose tissue (Wu et al., 2000). Wu et al. (2000) found that cells from different tissues responded to exogenous hormones differently with the greatest degree of hormonally-induced differentiation in subcutaneous fat. However, Grant and others saw no differential effects of troglitazone (TRO) on the differentiation of subcutaneous or intramuscular cells (2008a).

A majority of research on the regulation of adipogenesis has been conducted with murine models and *in vitro* studies using the immortalized 3T3-L1 cell line derived from disaggregated Swiss 3T3 mouse embryos (Green and Kehinde, 1974), although, some research has gone into creating and characterizing primary and clonally-derived bovine preadipocyte cell lines (Grant et al., 2008a and b; Taniguchi et al., 2008a and b). Classic hormones used to differentiate cells *in vitro* are isobutylmethylxanthine (IBMX), insulin, TRO, and dexamethasone (DEX). The first hormone listed, IBMX, is a phosphodiesterase inhibitor which increases cAMP and protein kinase A, which are part of an intracellular signaling cascade that ultimately lead to increased transcription factor activity including C/EBP-δ (Ntambi and Kim, 2000). Insulin acts through the insulin-like growth factor-1 receptor, which is a tyrosine kinase receptor, to stimulate transcription factor activity. Members of the thiazolindedione drug class, such as TRO, function as a PPARγ agonists (Kim et al., 2000; Grant et al., 2008b). Dexamethasone is a synthetic glucocorticoid that binds to a glucocorticoid receptor in the nucleus of the cell, or in cytosol of the cell and translocates to the nucleus (Ntambi and Kim, 2000). Once in the nucleus, the glucocorticoid-receptor complex homodimerizes and binds to the glucocorticoid response element on the DNA strand.

There are 2 general methods for isolating stromal-vascular (SV) cultures from bovine adipose tissue: enzymatic digestion and tissue explants. Each method has its own positives and negatives. For instance, enzyme digestion yields faster establishment of cultures, but tissue explants require less starting material and result in 'cleaner' cultures with less cellular debris. Fernyhough et al. (2005) detailed methodology of another

method of culture isolation where adipocytes, not preadipocytes, are specifically targeted. The basis of this approach is founded on the potential for adipocytes to 'de-differentiate', where mature, lipid-filled adipocytes revert back to a propogative state. The concept of de-differentiation disputes the idea that once cells accumulate lipid for storage, they are terminally differentiated. Aso et al. (1995) developed clonal preadipocyte cell lines with recombinant bovine fibroblast growth factor and differentiated cells with glucose, insulin, DEX, and acetate. Regardless of the isolation method, several laboratories have published papers pertaining to the gene expression and differentiation of bovine adipocytes *in vitro.* Grant et al. (2008a) demonstrated that bovine preadipocytes were capable of differentiating in response to combinations of insulin, serum lipids, DEX, and TRO. They also titrated an optimum level of TRO supplementation *in vitro* to induce differentiation in bovine primary SV cultures (2008b). Data used to support the occurrence of differentiation included Oil Red O staining of lipid in cells and increased glycerol-3-phosphate dehydrogenase (GPDH) protein and activity. Hirai et al. (2007) also reported on SV-culture isolation via collagenase digestion and differentiation of cultures. Using insulin, IBMX, TRO, and DEX, they reported successful differentiation of cultures evidenced by Oil Red O staining, GPDH activity, Northern blot of aP2 mRNA expression, and semi-quantitative PCR of C/EBP-α,-β and PPARγ mRNA. In another study, insulin, IBMX, and DEX were used to initiate differentiation and followed by the addition of intralipid or lipoproteins, which enhanced differentiation in cultures (Wu et al., 2000). Lengi and Corl (2010), who use the tissue explant method of SV isolation, tested several different reported hormone media for their efficacy to induce

differentiation. The objective measures of differentiation used were radiolabeled acetate incorporation (1,2-^{14}C2) into cellular lipids, GPDH activity, and mRNA expression of aP2, PPARγ, and ACC (Lengi and Corl, 2010). All media combinations tested positive for at least parameter of differentiation compared with the no hormone-added controls. Expression of aP2 and PPARγ mRNA was elevated with the Aso et al. (1995) and Wu et al. (2000) media; whereas, ACC mRNA expression and acetate incorporation were elevated with the Wu et al. (2000) and Hirai et al. (2007) media. Addition of TRO or roglitazone, another member of the thiazolidinediones drug class, to the Wu et al. (2000) media further enhanced differentiation in all parameters tested (Lengi and Corl, 2010). Despite the amount of literature on differentiation and gene expression, no fatty acid composition data has been reported in bovine adipocyte cultures to date. As lipogenic genes are activated during the course of differentiation, the fatty acid composition may be subject to change also.

FATTY ACID REGULATION

Regulation of *de novo* fatty acid synthesis can occur at many different levels: transcription of mRNA, translation of message into protein, and activity of the protein. Each enzyme in the lipogenesis pathway can be regulated (Table 2), in addition to cellularity of adipose tissue. The first enzyme in fatty acid synthesis acetyl-CoA carboxylase (ACC) is regulated allosterically and by phosphorylation. Citrate activates ACC by changing its conformation to a multimeric, filamentous complex, and palmitoyl-CoA inactivates ACC by promoting a monomeric, or protomer, form. Insulin-activated protein phosphotase activates ACC by dephosphorylation, and AMP-activated kinase inactivates it by phosphorylation. Regulation of ACC can also occur at the level of transcription, but is not the primary mechanism. The next enzyme responsible for *de novo* fatty acid synthesis, FASN, is transcriptionally regulated. Insulin stimulates its expression by mediating upstream regulatory factors and SREBP (Eberlé et al., 2004). Leptin diminishes its expression inhibiting SREBP and directly blocking FASN transcription. Regulation of SCD is predominantly at the transcription level; although, it can be directly inhibited by sterculic acid, a cyclopropenoid fatty acid (Gomez et al., 2003). Expression of SCD is sensitive to many dietary factors, hormones, and SREBP-1c activity (Tabor et al., 1999; Ntambi and Miyazaki, 2004). The elongase proteins are also thought to be regulated at the transcription level (Matsuzaka et al., 2002). Transcription factors that regulate ELOVL6 include SREBP and PPARγ; ELOVL5 expression is up-regulated PPARγ and not by SREBP (Wang et al., 2006). Allosteric regulation of

ELOVL6 is also being investigated using a synthetic, chemical inhibitor (Shimamura et al., 2009; Shimamura et al., 2010).

Stearoyl-CoA desaturase is the rate-limiting enzyme in the production of MUFA from SFA. It is one of several markers for differentiation of preadipocytes into mature adipocytes, along with aP2 and PPARγ (Chawla et al., 1994; Ohsaki et al., 2007). Stearoyl-CoA desaturase is called a Δ^9 desaturase because it inserts a *cis*-double bond between the 9^{th} and 10^{th} carbons in the hydrocarbon skeleton, counting from the carboxyl end (Ntambi, 1999). The preferred substrates for SCD are palmitoyl- and stearoyl-CoA, which become palmitoleoyl- and oleoyl-CoA, respectively. Also, SCD can convert *trans*-vaccenic acid (C18:1 *trans*-11), a biohydrogenation intermediate, to CLA *cis*-9, *trans*-11, a potent anticarcinogen (Parodi, 1997). Because of its role in biosynthesis of MUFA, SCD can impact membrane fluidity and composition of stored triglycerides by changing the ratio of SFA: MUFA. While transcription of SCD mRNA is thought to be controlled primarily by SREBP that binds to sterol response element in the promoter region of the SCD gene, it is also regulated by many other factors (Eberle et al., 2004; Lay et al., 2002). As reviewed by Ntambi and Miyazaki (2004), glucose, cholesterol, vitamins A and D, insulin, and estrogen increase SCD expression, but PUFA, leptin, glucagon, and thiazolidinediones decrease it. In addition to PUFA's control over SCD expression, SCD is regulated in part by its products and not by the availability of its substrates (Keating et al., 2006). For instance, the addition of CLA *cis*-9, *trans*-11 reduces expression of SCD mRNA, but increasing amounts of C18:1 *trans*-11 does not. The promoter region of the SCD gene is thought to contain a fat specific element, PUFA

response element, and SREBP-response region (Keating et al., 2006). Decreased SCD expression seen in murine and mammary cells treated with C20:4 n-6 and CLA *trans*-10, *cis*-12 may be a result of altered promoter binding at the PUFA response element of the SCD gene.

The majority of research focused on SCD has used the rodent model or the immortalized cell line 3T3-L1. However, in contrast to the localized, hepatic-expression of murine SCD, bovine SCD is expressed throughout the body in adipose tissue of growing animals and also in the mammary gland of lactating animals (Martin et al., 1999; St John et al., 1991). Four isoforms of SCD have been identified in mice and, thus far, only two isoforms have been identified for SCD in ruminant species (Lengi and Corl, 2007; Ward et al., 1998). The SCD1 isoforms in cattle, goats, and sheep are homologs and similar to murine SCD1; the SCD5 isoform, once thought to be unique to primates, is expressed in the brain and pancreas of humans and cattle (Lengi and Corl, 2007; Wang et al., 2005). Despite inherent differences in ruminant adipose tissue metabolism that exist compared to monogastric animals, in terms of glucose uptake and *de novo* synthesis of fatty acids from acetate (Aso et al., 1995), they are similar with respect to location of SCD isoform expression.

There is also a relationship between SCD expression and obesity. High levels of SCD are seen in obese human patients, as well as the insulin-resistant, obese Zucker rats (Hulver et al., 2005; Voss et al., 2005). It remains to be determined if elevated SCD is the causal agent in adiposity or if increased SCD is simply a product of greater abundance of white adipose tissue in obese animals. Therefore, investigation of SCD

over-expression may lead to valuable insights into the role of SCD *in vivo*, especially in regards to tumor development, obesity, and type II diabetes. Stearoyl-CoA desaturase over-expression has been associated with increasing MUFA, decreasing SFA, and subsequent weakening of cellular membranes (Sun et al., 2003). With increasing amounts of SCD, the primary products of its enzymatic reaction, C18:1 *cis*-9 and C16:1, increase, provided that substrates are available. Increasing ratios of C18:1 *cis*-9/C18:0 are associated with certain types of tumors, thus implicating SCD in carcinogenesis (Khoo et al., 1991). In particular, high levels of SCD have also been related to several conditions that are directly linked with hepatocarcinogenesis in humans, including hepatosteatosis (Falvella et al., 2002). Research involving over-expression of SCD in adipocytes is limited, due in part to the difficulties associated with transfecting fat cells. Gene knock-down and silencing techniques have been used most often to investigate the role of SCD.

Prime animal models for the study of SCD are the SCD1-deficient asebia mice and SCD1-knockout mice. Some of the effects of SCD knockdown in mice are visually evident. Mice with targeted disruption of the SCD1 gene are leaner than their wildtype counterparts and resistant to diet-induced adiposity (Ntambi et al, 2002). Whole gene deletions of SCD1 in mice also result in a lean phenotype, but have poor hair coat and dry eyelids due to atretic sebaceous and meiobium glands thought to be caused by increased circulation of free cholesterols previously associated with cell death (Ntambi and Miyazaki, 2004). Other non-visible characteristics of SCD1 depression are increased insulin sensitivity, glucose transporter 4 (GLUT4) receptors, and metabolic rate (Rahman

et al., 2003; Ntambi et al., 2002). Differentiation of non-ruminant preadipocytes increases insulin-stimulated glucose uptake by GLUT4 (Kaestner et al., 1989). In mice, SCD knockout increases the levels of GLUT4 in the cellular membranes (Rahman et al., 2003). However, in bovine preadipocytes and adipocytes, Aso et al. (1995) reported that GLUT4 was not active. To date, SCD1 or SCD5 knockdown has not been performed on bovine adipocytes. If SCD is absent, biosynthesis of MUFA from SFA cannot proceed, and SFA will accumulate in the cell, potentially causing cell death due to lipotoxicity. Lipotoxicity, an event that triggers apoptosis, has been associated with high levels of SFA and can be attenuated by supplementation with unsaturated fatty acids (Hardy et al., 2000; Listenberger et al., 2003). In murine hepatocytes, C16:0 induced apoptosis, but C18:1 *cis*-9 rescued cells from C16:0-induced apoptosis and promoted lipid filling in hepatocytes (Li et al., 2009).

Similar to results seen with SCD1-deficient mice, aP2-deficient mice also show protection against diet-induced obesity, reduced hepatosteatosis, and improved insulin sensitivity (Hotamisligil et al., 1996; Newberry et al., 2006). These are interesting systemic effects as expression of this lipid chaperone, as well as SCD1 expression, is very tissue specific. In addition, there seems to be depot-specific regulation of lipogenic genes as mRNA expression of ACC, FASN, SCD1, and ELOVL6 were all down-regulated in liver and up-regulated in the epididymal fat pad of aP2-knockout mice (Cao et al., 2008). Analysis of lipid profiles in these tissues revealed palmitoleic acid (C16:1 *cis*-9), a product of the SCD1 reaction, was significantly elevated in aP2-knockout compared to wildtype mice for all lipid classes analyzed: free fatty acids, triglycerides,

phospholipids, and cholesterol esters (Cao et al., 2008). When plasma lipids from aP2-knockout mice were used to incubate adipose tissue explants, Cao et al. (2008) reported a decrease in inflammatory properties. Furthermore, murine adipocytes incubated with palmitoleic acid showed a reduction in cytokine expression compared with adipocytes incubated with palmitic acid (C16:0). Murine SV cultures did not show differences due to fatty acid treatment suggesting palmitoleic acid affects functional adipocytes only (Cao et al., 2008). These results led to the hypothesis that dysfunction of lipid metabolism seen in disease states associated with obesity could be attenuated by exogenous palmitoleic acid administration; that palmitoleic acid could act as a hormone signal to the rest of the body. To summarize the results of Cao et al. (2008) produced by several different methodologies, palmitoleic acid decreased hepatic SCD1 mRNA and protein expression, decreased FASN and ELOVL6 mRNA expression, and increased skeletal muscle insulin sensitivity and glucose disappearance from plasma. Based on this data, Cao et al. (2008) suggested palmitoleic acid may function as a hormone and coined the term 'lipokine'. However, the mode of regulation is remains unclear. Scrambling the sterol response element in the promoter region of the SCD gene removed palmitoleic acid's inhibitory effect on SCD mRNA expression (Cao et al., 2008). Therefore, the mechanism through which palmitoleic acid could be acting is SREBP-1c. As stated previously, SREBP binds to the sterol response element of several lipogenic genes to affect their transcription. In addition, palmitoleic acid could be acting more directly on lipogenic genes at the protein level. The half-life of SCD1 is known to be relatively short. Using a Flag-tagged SCD1 under the cytomegalovirus promoter, palmitoleic acid

supplementation increased SCD1 degradation in hepatocytes; whereas, palmitic acid stabilized it (Cao et al., 2008).

Overall, the lipokine hypothesis highlights a potential regulatory pathway in which palmitoleic acid functions as an indicator of energy status in the body. Palmitoleic acid is usually present at low levels throughout bodily tissues and is primarily produced by *de novo* fatty acid synthesis, as there are very few dietary sources high in palmitoleic acid. Acetyl-CoA carboxylase and FASN are responsible for assimilating malonyl-CoA and fatty acyl-CoA into C16:0 and C18:0 fatty acids from acetate or glucose precursors. Both C16:0 and C18:0 can be desaturated by SCD into C16:1 *cis*-9 and C18:1 *cis*-9, respectively. A build-up of palmitoleic acid would suggest to the body that energy sources are abundant, fatty acid synthesis and desaturation are proceeding too rapidly, and resources should be partitioned away from lipogenesis, perhaps toward gluconeogenesis, glycogen synthesis, or β-oxidation. In humans, high plasma concentrations of palmitoleic acid are associated with elevated serum triglycerides, abdominal adiposity, and overall obesity (Attie et al., 2002). Additionally, an observational study by Gong et al. (2010) reported a positive association between palmitoleic acid in adipose tissue and obesity. A low carbohydrate diet reduced this correlation (Gong et al., 2010). It is possible that fatty acid composition in the diet, plasma, liver, and adipose tissue depots may all contribute to regulating lipid and carbohydrate homeostasis. Research in this area is limited due to inherent complexities of controlling for diverse physiologic and pathological conditions which can impact lipid composition and metabolism. If lipogenesis could be limited to certain depots of adipose

tissue and glucose metabolism in skeletal muscle improved with the strategic use of palmitoleic acid, it might serve as therapy for obesity and type II diabetes in humans, as well as a tool for improved carcass characteristics and production efficiency in animal agriculture.

Table 1. Predominant fatty acids in ruminant tissues: Nomenclature, primary sources, and implications for human health.

Fatty Acid Nomenclature				
Common Name	Lipid Numbers	IUPAC Name	Primary Source in Ruminants[1]	Implications for Human Health
Palmitic acid	C16:0	hexadecanoic acid	*de novo* synthesis	raises serum LDL[2]
Palmitoleic acid	C16:1 *cis*-9	(Z)-9-hexadecenoic acid	desaturated from C16:0	associated with obesity and insulin[3]
Stearic acid	C18:0	octadecanoic acid	biohydrogenation	lower serum LDL[4]
Oleic acid	C18:1 *cis*-9	(Z)-9-octadecenoic acid	desaturated from C18:0	lower LDL and blood pressure[5]
Cis-Vaccenic acid	C18:1 *cis*-11	(Z)-11-octadecenoic acid	biohydrogenation, elongated from C16:1	lower total cholesterol and TG[6]
[*Trans*-] Vaccenic acid	C18:1 *trans*-11	(E)-11-octadecenoic acid	biohydrogenation	precursor of CLA *cis*-9, *trans*-11
Linoleic acid	C18:2 n-6	(Z,Z)-9,12-octadecadienoic acid	dietary by-pass	essential fatty acid[7]
Conjugated linoleic acid (CLA)	C18:2 *trans*-10, *cis*-12	(E,Z)-10,12-octadecadienoic acid	biohydrogenation	potential anti-obesity[8]
Rumenic acid (or CLA)	C18:2 *cis*-9, *trans*-11	(Z,E)-9,11-octadecadienoic acid	biohydrogenation, desaturated from C18:1 *trans*-11	potential anti-carcinogenic[9]
[α-]Linolenic acid	C18:3 n-3	(Z,Z,Z)-9,12,15-octadecatrienoic acid	dietary by-pass	essential, anti-inflammatory[7]
Arachidonic acid	C20:4 n-6	(Z,Z,Z,Z)-5,8,11,14-eicosatetraenoic acid	dietary by-pass, synthesis from C18:2n-6	essential, inflammatory mediator[7]

[1]Primary source of endogenous fatty acids found in ruminant tissues.

[2]Hegsted et al., 1965

[3]Cao et al., 2008; Gong et al., 2010; Stefan et al., 2010

[4]Denke and Grundy, 1985

[5]Mattson and Grundy, 1985

[6]Uchiyama et al., 1966

[7]Burr et al., 1932

[8]Park et al., 1999

[9]Parodi et al., 1997

Table 2. Lipogenic gene function and regulation.

Gene Name	Function	Mode of Regulation	Known Inhibitors (Activators)
Acetyl-CoA Carboxylase (ACC)	first step of *de novo* lipogenesis	allosteric inhibition	C16:0 (Citrate)
		phosphorylation	AMP-activated kinase (Insulin-activated protein phosphotase)
		transcription	
Fatty acid synthase (FASN)	*de novo* lipogenesis up to 16C	transcription	Leptin (Insulin, SREBP-1c)
Stearoyl-CoA desaturase 1 (SCD1)	inserts double bond at Δ^9 position	transcription	PUFA (Insulin, SREBP-1c)
		allosteric inhibition	Sterculic acid
Fatty acid elongase 5 (ELOVL5)	adds 2C to fatty acids C in length	transcription	(Peroxisome proliferator-activated receptor γ (PPARγ))
Fatty acid elongase 6 (ELOVL6)	adds 2C to fatty acids 16-18C in length	transcription	(SREBP-1c, PPARγ)
		allosteric inhibition	synthetic drug
Sterol regulatory element binding protein (SREBP-1c)	lipogenic gene transcription factor	proteolytic maturation	
Carnitine palmitoyl-transferase 1A (CPT1A)	transport of fatty acids for β-oxidation	allosteric inhibition	Malonyl-CoA
		transcription	

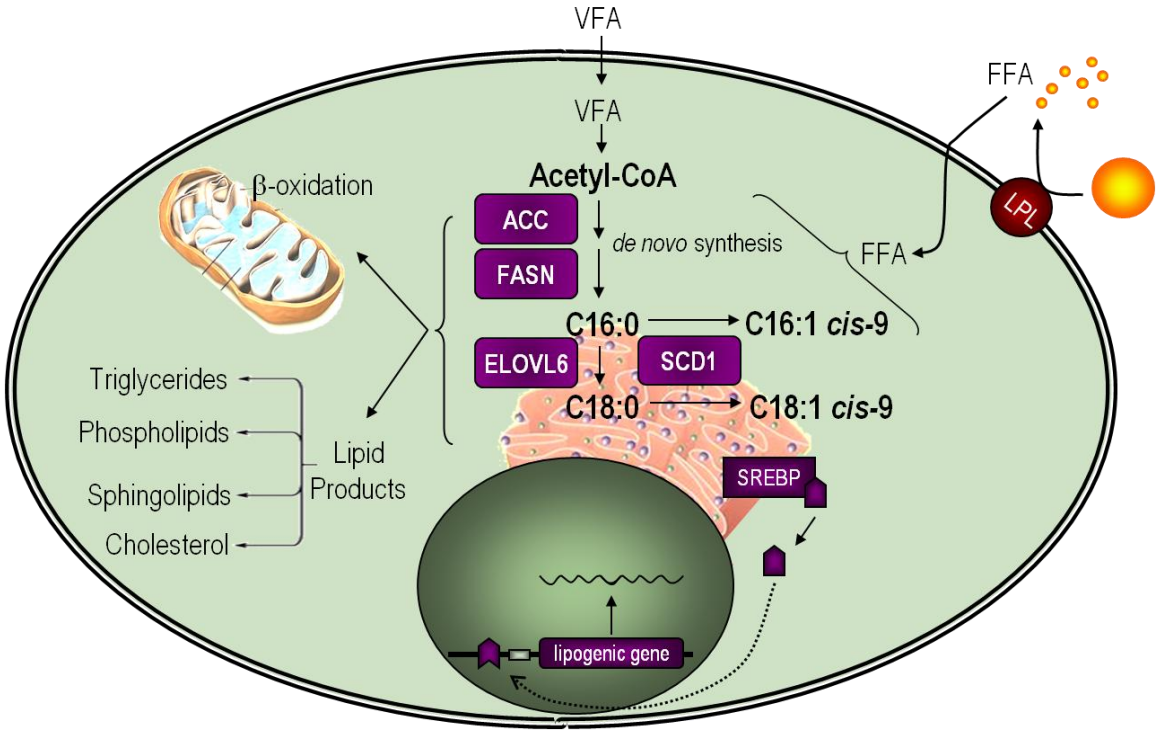

Figure 1. An overview of fatty acid synthesis in the ruminant adipocyte. Lipids packaged in lipoproteins are transported to adipocytes where lipoprotein lipase (LPL) releases free fatty acids, which can diffuse through the cell membrane. Free fatty acids can undergo fatty acid synthesis reactions such as desaturation by stearoyl-CoA desaturase 1 (SCD1). Volatile fatty acids (VFA) can freely diffuse across the cell membrane and undergo *de novo* fatty acid synthesis. Acetyl-CoA carboxlyase (ACC) is the committed step, followed by sequential fatty acid synthase (FASN) reactions which elongate fatty acyl-CoA to 16C in length. Bound to the endoplasmic reticulum membrane, elongase protein 6 (ELOVL6) can further elongate fatty acyl-CoA and SCD1 can add a double bond at the Δ^9 position. Fatty acids can be packaged for use or storage into other lipid products or undergo β-oxidation in the mitochondria for energy production. Sterol regulatory element binding protein-1c (SREBP) is a transcription factor for many lipogenic genes. Other proteins, sensing intracellular signals, carry SREBP to the golgi apparatus where it is proteolytically matured. Once in the active form, SREBP travels to the nucleus where it serves as a transcription factor, binding to the promoter region of several lipogenic genes.

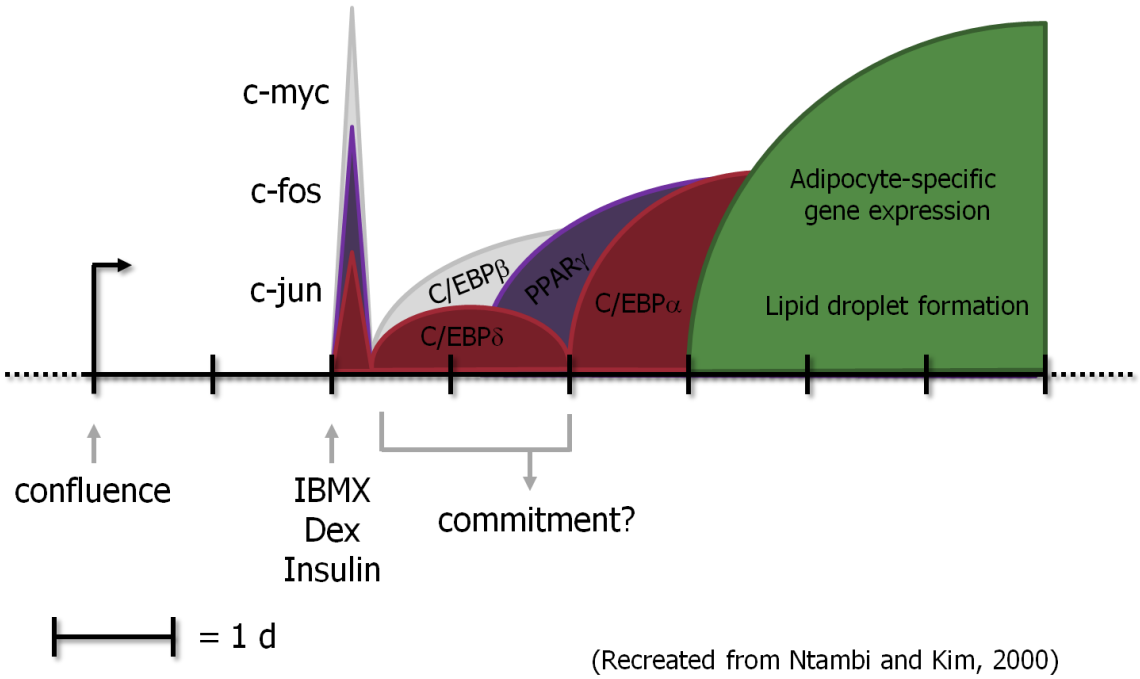

Figure 2. Chronological progression of major gene expression changes in differentiation of 3T3-L1 cells as reviewed by Ntambi and Kim (2000).

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CHAPTER 2: GENE EXPRESSION AND FATTY ACID PROFILES DURING DIFFERENTIATION OF BOVINE ADIPOCYTE CULTURES SUPPLEMENTED WITH LINOLEIC ACID (C18:2 n-6)

ABSTRACT

The primary objectives of this study were to determine if fatty acid profiles or lipogenic gene expression differ when *in vitro* adipocytes are exposed to supplemented linoleic acid (C18:2 n-6), insulin, or both following differentiation. With limited information in the literature about the timing of lipid uptake and composition of fatty acids in differentiating bovine adipocytes, a secondary objective of this study was to evaluate fatty acid composition over time. Primary stromal vascular cultures were isolated from 18 mo. old Angus crossbred steers by collagenase digestion. In the first experiment, cells were hormonally induced to differentiate on d 0, cultured to d 12, serum starved for 24 h, and assigned to 1 of 4 treatments in a 2 X 2 factorial design consisting of linoleic acid (0 mM or 0.3 mM C18:2 n-6) and insulin (0 or 2.5 μg/mL) as factors. Cells were harvested on d 18, following 5 d of incubation with respective treatments, for fatty acid analysis and gene expression. In a second experiment, cells were assigned to treatments according to a 2 X 3 factorial design consisting of linoleic acid (0 or 0.3 mM C18:2 n-6; beginning on d 2) and day of harvest (d 2, 6, or 12) as factors. Undifferentiated cells harvested on d 2 served as baseline controls. As before, cells were harvested for fatty acid and gene expression analysis. In the first experiment, total fatty acids and C18:2 n-6 increased ($P < 0.001$) and palmitic (C16:0), stearic (C18:0), oleic

(C18:1 *cis*-9), and linolenic (C18:3 n-3) acids decreased ($P < 0.05$) due to a main effect of linoleic acid supplementation. Desaturation indices and SCD1 mRNA expression also decreased ($P < 0.05$) in response to linoleic acid supplementation. Insulin treated cells also had decreased ($P < 0.05$) C18:1 *cis*-9/C18:0 desaturation ratio compared with 0 μg/mL treated cells and tended ($P = 0.08$) increased C18:2 n-6. In the second experiment, there was interaction ($P < 0.05$) of linoleic acid supplementation and day of harvest for major fatty acids and desaturation indices. With the exeption of C16:0, individual fatty acids and the ratio of C18:1 *cis*-9/C18:0 increased over time in unsupplemented cells and decreased or remained unchanged on d 6 and 12 in linoleic acid-treated cells. Regardless of linoleic acid supplementation level, analysis of mRNA expression revealed that fatty acid synthase (FASN), glucose transporter 4 (GLUT4), and peroxisome proliferator-activated receptor gamma (PPARγ) mRNA expression was elevated ($P < 0.05$) in differentiated cells on d 12 compared with undifferentiated cells. Messenger RNA of stearoyl-CoA desaturase 1 (SCD1) and adipocyte protein 2 (aP2), differentiation-associated genes, was up-relgulated ($P < 0.05$) in differentiated cells on d 2 compared with undifferentiated cells and down-regulated ($P < 0.05$) on d 6 and 12 compared with d 2. A significant interaction existed between time and treatment for sterol regulatory element binding protein-1c (SREBP). Unsupplemented cultures were decreased ($P < 0.05$) in their expression of SREBP mRNA on d 6 and 12 compared with undifferentiated cells; whereas, linoleic acid-treated cells increased ($P < 0.05$) over time. In conclusion, fatty acid composition changes with respect to time following induction of differentiation and also reflects changes in the composition of the media. Supplementing

culture media with linoleic acid reduced SCD1 transcription at d 18 and decreased desaturation indices at d 6, 12, and 18. Bovine adipocyte cultures were actively desaturating fatty acids, which led to changes in fatty acid composition of the cultures. In addition, by d 6 cultures were sensitive to lipogenic regulation by exogenous fatty acid administration. Based on our data of fatty acid uptake and mRNA expression changes of differentiation-associated genes, we propose to time future investigations of bovine adipocytes more closely with differentiation, from d 6 to 12, when cells are fully functional adipocytes.

INTRODUCTION

In the beef industry, USDA grading standards estimate cutability and eating quality of the lean tissue to determine carcass value. These yield and quality grades applied to beef carcasses are highly influenced by the abundance of subcutaneous and intramuscular fat, respectively. During the finishing stage in cattle, fat deposition in the form of white adipose tissue is the result of both hyperplasia and hypertrophy (Hood and Allen, 1973). Although cell number and size both increase during animal maturation, 70% of fat accumulation is the result of hypertrophy (Robelin et al., 1985). Preadipocytes, cells responsible for hyperplasia, are of mesenchymal lineage and have the ability to propagate or commit to differentiation (Gesta et al., 2007). Upon stimulation, they differentiate into mature adipocytes, which have the ability to synthesize, take up, and store fat. The composition and quantity of white adipose tissue is influenced by animal nutrition. Cattle finished on high concentrate diets have altered fatty acid profiles and lipid deposition rates compared to pasture-finished animals (Pavan and Duckett, 2008). Therefore, diets of finishing cattle can potentially impact carcass value by altering adipose tissue at the level of differentiating preadipocytes, lipid-filling adipocytes, or both.

Over the past decade, researchers have begun to view white adipose tissue, not as an inert energy storage site, but as a complex, metabolically active tissue (McGillis, 2005). Obesity is associated with many common medical disorders including cardiovascular disease and type II diabetes. The amount and location of white adipose tissue deposits affect disease risk in humans (Gesta et al., 2007). Health professionals and

consumers are increasingly concerned about saturated fatty acids (SFA) in beef. By defining mechanisms through which adipogensis and lipogenesis are controlled, we can improve the nutritive quality of beef and potentially develop models to enhance our understanding individual health and obesity-onset in humans.

A majority of research in fatty acid metabolism and adipogenesis has been conducted with murine models and *in vitro* studies using the immortalized 3T3-L1 cell line derived from disaggregated Swiss 3T3 mouse embryos (Green and Kehinde, 1974), although some research has gone into creating and characterizing primary and clonally-derived bovine preadipocyte cell lines (Grant et al., 2008a and b; Taniguchi et al., 2008a and b; Lengi and Corl, 2010). Gene expression during preadipocyte differentiation has been well documented in 3T3-L1 cells (Ntambi and Kim, 2000). To date, information regarding early events in bovine preadipocyte differentiation is primarily limited to gene expression. Inherent differences in site of lipogenesis and substrate utilization in murine versus ruminant species necessitate investigation in this area. Our objective in this study was to evaluate the fatty acid profiles and the expression of genes involved in differentiation and lipogenesis in bovine preadipocytes. Fat is often added to livestock diets as energy supplements and in vitro cultures are sometimes used to investigate *in vivo* effects. Linoleic acid (C18:2 n-6) is an essential fatty acid and is the predominate fatty acid found in most livestock feedstuffs and commonly used fat supplements, such as corn oil. Duckett et al. (2009) reported up-regulated stearoyl-CoA desaturase 1 (SCD1) mRNA expression in adipose tissue of steers fed a corn-based supplement. Insulin is associated with high-carbohydrate diets and SCD1 activity (Vessby et al., 2002; Flowers

and Ntambi, 2009). We hypothesized that supplementing bovine adipocytes cultures with linoleic acid, insulin, or both would impact gene expression and fatty acid composition.

MATERIALS AND METHODS

Cell Culture. Primary bovine stromal vascular (SV) cultures were harvested from subcutaneous flank fat of 18 mo old Angus crossbred steers according to Hirai et al. (2007). At slaughter, ~30 cm^3 sections of adipose tissue were excised and minced using sterile instruments, rinsed with Hank's Balanced Salt Solution (HBSS), and digested in 25 mL HBSS containing 2 mg/mL collagenase, type I, and 40 mg/mL bovine serum albumin under constant shaking at 37°C for 120 min. Remaining tissue fragments and fat-filled adipocytes were separated from the stromal vascular fraction by filtering through a 250 μm nylon mesh and subsequent centrifugation at 2000 x g for 10 min. Cells were plated at 1 x 10^4 cells/cm^2 and passaged every 2 to 4 d when approximately 60% confluent. Cells were incubated at 37°C under 5% CO_2 humidified atmosphere with media [Dulbecco's modified eagles medium (DMEM) containing 10% fetal calf serum (FCS), and 2X antibiotiotic/antimycotic (AB/AM; containing 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B)] replaced every 2 d. After 4 passages, cell cultures were harvested and stored in liquid nitrogen at 1 x 10^6 cells/mL in freeze media (DMEM, 20% FCS, and 10% dimethyl sulfoxide) for later use.

Experiment 1. Individual SV cultures from 3 steers were used in this study. Cells were thawed, passaged 3 times, and seeded in plates at 1 x 10^5 cells/cm^2. Cells were allowed to reach confluence, held for 2 d, and differentiated on d 0 according to Hirai et al. (2007) with DMEM containing 5% FCS, 2X AB/AM, insulin at 2.5 μg/ml, 0.5 mM 2-isobutyl-1-methylaxanthine (IBMX), 0.25 μM dexamethasone (DEX), and 5 μM troglitazone (TRO). Secondary differentiation media (DMEM, 5% FCS, 2X AB/AM,

insulin at 2.5 μg/mL, 5 μM TRO) was applied, beginning on d 2 to d 12. On d 12, the secondary differentiation media was replaced by a starvation media (DMEM, 0.1% BSA, 2x Ab/Am) for 24 h. Treatments, performed in duplicate for each culture, were applied in a 2 X 2 Factorial design with bovine insulin (0 μg/mL or 2.5 μg/mL; Sigma-Aldrich, St. Louis, MO) and linoleic acid (0 mM or 0.3 mM C18:2 n-6; BD Biosciences, Bedford, MA) as supplements in culture media. Cells were harvested on d 18 for fatty acid and gene expression analysis by washing 3 times with PBS followed by trypsin digestion.

Experiment 2. Individual cultures from 2 steers were passaged and plated as described above. Cells were allowed to reach confluence, held for 2 d, and differentiated on d 0 as described by Hirai et al. (2007) DMEM containing 5% FCS, 2X AB/AM, insulin at 2.5 μg/ml, 0.5 mM 2-isobutyl-1-methylaxanthine (IBMX), 0.25 μM dexamethasone (DEX), and 5 μM troglitazone (TRO) followed by media modifications described by Pratt et al. (2010). Secondary differentiation media (DMEM, 5% FCS, 2X AB/AM, insulin at 2.5 μg/mL, 5 μM TRO) supplemented with 0 mM or 0.3 mM linoleic acid was applied from d 2 to 12. Undifferentiated cells were harvested on d 2 and differentiated cells supplemented with or without linoleic acid were harvested on d 2, 6, and 12 for fatty acid and mRNA expression analysis. All treatments were performed in duplicate for each SV culture.

Fatty acids. Cellular fatty acids were extracted as described by Folch et al. (1957) and transmethylated according to Park and Goins (1994). Fatty acid methyl esters were analyzed using an Agilent 6850 gas chromatograph equipped with an Agilent 7673A automatic sampler (Agilent Technologies, Inc., Santa Clara, CA). Separations

were accomplished using a 100-m Supelco SP-2560 (Supelco, Inc., Bellefonte, PA) capillary column (0.25 mm i.d. and 0.20 μm film thickness) according to Duckett et al. (2002). Individual fatty acids were identified by comparison of retention times with standards (Sigma, St. Louis, MO; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating an internal standard, methyl tricosanoic (C23:0) acid, into each sample during methylation and expressed as a weight percentage of total fatty acids per well.

Gene Expression. Total cellular RNA was isolated from cells using the *mir*Vana microRNA Isolation kit (Ambion, Austin, TX) according to manufacturer's instructions and RNA quality as described by Duckett et al. (2009). Quality was assessed using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA); tcRNA samples used in real-time PCR (qRT-PCR) had a 260:280 absorbance ratio > 1.7. Real-time qRT-PCR was conducted using an Eppendorf MasterCycler ep realplex (Westbury, NY) with the QuantiTect SYBR Green RT-PCR One Step Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Two genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, were evaluated as housekeeping genes for data normalization (Duckett et al., 2009). To determine the appropriate housekeeping gene to be used to normalize the data, the cycle threshold values (C_T) for GAPDH, β-actin, and all target genes per sample were entered into the BESTKEEPER program (http://www.gene-quantification.info). The program determines the most stable housekeeping gene to be used for normalization by repeated pair-wise correlation and regression analysis (Pfaffl et al., 2002). Both GAPDH and β-actin exhibited a correlation

coefficient of 0.99 ($P < 0.001$) in the analysis and were suitable for data normalization. Primers for bovine mRNAs were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Primer sets were first evaluated according to Duckett et al. (2009) to verify identity. Genes of interest were those involved in fatty acid biosynthesis, acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), and SCD1, as well as their transcription factors, peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element binding protein (SREBP). Additionally, glucose transporter 4 (GLUT4) was gene expression was analyzed.

Statistical Analysis. The data were analyzed using Proc GLM procedure of SAS 9.2 (Cary, NC) for treatment comparisons over time. A two-way analysis of variance (ANOVA) was performed as a completely randomized design with factors consisting of insulin (0 or 2.5 μg/mL) and linoleic acid (0 or 0.3 mM C18:2 n-6) for Experiment 1 and linoleic acid (0 or 0.3 mM C18:2 n-6) and day of differentiation (d 2, 6, or d 12) for Experiment 2. Least squares means are computed and separated statistically using Fisher's Protected LSD test. When the overall *F*-test for the treatment effect was significant ($P \leq 0.05$), a *t*-test was performed to discern the differences among treatments by using the PDIFF option of the LSMeans statement. For gene expression data, normalized C_T values were subjected to ANOVA, as described above. Additionally, relative gene expression data was analyzed using Pair-wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002) with preplanned treatment comparisons.

RESULTS AND DISCUSSION

The differentiation hormones and media used in our experiment have been tested for their efficacy in inducing differentiation in bovine SV cultures (Aso et al., 1995; Grant et al., 2008a and b; Lengi and Corl, 2010). Classically, fibroblastic preadipocytes change shape from stellate to round and accumulate lipid (Napolitano, 1963), in addition to changes in gene expression (Taniguchi et al., 2008a). Previously, our SV cultures displayed characteristic changes in morphology, lipid-filling, and adipogenic gene expression upon hormonal treatment, consistent with adipocyte differentiation (Pratt et al., 2010). In the current study, cells changed shape from fibroblastic to round following differentiation and became lipid-filled, especially with fatty acid supplementation (data not shown).

Experiment 1. There was a main effect of linoleic acid supplementation on palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1 *cis*-9), C18:2 n-6, linolenic acid (C18:3 n-3), and total fatty acid levels (Figure 1). Each fatty acid identified, with the exception of C18:2 n-6, decreased ($P < 0.05$) in response to linoleic acid supplementation. This suggests that the presence of abundant PUFA could decrease lipogenesis or stimulate β-oxidation of endogenous fatty acids. Insulin supplementation did not affect ($P > 0.05$) fatty acid composition, although there was a trend ($P = 0.07$) for increased C18:2 n-6 in insulin-treated cultures.

Stearoyl-CoA desaturase 1 (SCD1) is the enzyme responsible for creating a double bond at the Δ^9 position of several fatty-acyl CoA and its preferred substrates are C16:0 and C18:0 (Enoch et al., 1976). Its transcription and activity can regulated by

certain fatty acids, including linoleic acid (Ntambi, 1995). The level of linoleic acid supplementation used in this study was based on a dose titration by Sessler et al. (1996) for arachidonic (C20:4 n-6) inhibition on SCD1. They tested the effects of several fatty acids on SCD1 mRNA expression in 3T3-L1 adipocytes, an immortalized murine cell line. In addition to arachidonic acid, 0.3 mM linoleic acid and linolenic (C18:3 n-3) acids reduced SCD1 mRNA expression (Sessler et al., 1996). In our study, we also found SCD1 mRNA expression to be down-regulated in linoleic acid supplemented cells (Figure 2), in addition to desaturation indices (Figure 3).

Experiment 2. Indicative of functional adipocytes cultures, total cellular fatty acids increased ($P < 0.001$) over time in both groups, but to a greater extent in linoleic acid-treated cells ($P < 0.001$, Table 1). Linoleic acid incorporation into the cells was demonstrated by increased ($P < 0.01$) C18:2 n-6 in linoleic acid-treated cells compared with unsupplemented on both a percent and gravimetric basis on d 6 and d 12 (Table 1 and 2). Corresponding with increased C18:2 n-6 in linoleic acid-treated cells, C16:0, C18:0, C18:1 *cis*-9, and C18:3 n-3 fatty acids decreased ($P < 0.001$) on a percent basis for both d 6 and d 12 compared with unsupplemented cells (Table 2).

Predominate fatty acids of interest in this study were products of *de novo* fatty acid synthesis, C16:0 and C18:0, products of desaturation, C16:1 and C18:1 *cis*-9, and PUFA, C18:2 n-6 and C18:3 n-3. For each of these fatty acids, there was a significant interaction ($P > 0.05$) between linoleic acid supplementation and day, indicating that fatty acid composition changed over time within differentiating bovine adipocytes and linoleic acid supplementation affected how that composition changed over time. The most

obvious examples of this are the monounsaturated fatty acids (MUFA), C16:1 and C18:1 *cis*-9. Both MUFA were increased ($P < 0.05$) on a gravimetric basis by d 6 and were greatest in d 12 in unsupplemented cells; in linoleic acid-treated cells, however, MUFA did not change over time ($P > 0.05$, Table 1). The desaturation indices of the bovine adipocytes cultures followed a similar pattern. Both MUFA/SFA and C18:1 *cis*-9/C18:0 indices increased ($P < 0.01$) over time for unsupplemented cells, but decreased ($P < 0.05$) over time in linoleic acid-treated cells. The C16:1/C16:0 ratio also followed this trend numerically, however, was not statistically significant.

Gene Expression. Messenger RNA levels of SCD1, aP2, and PPARγ, differentation-associated genes, did not differ ($P > 0.05$) due to linoleic acid supplementation compared with unsupplemented cells. An indicator of differentiation and desaturation, SCD1 mRNA expression was up-regulated ($P < 0.05$) 2 d after differentiation media was applied. From d 2 to d 6 SCD gene expression decreased ($P < 0.05$), but then up-regulated ($P < 0.05$) to an intermediate level between d 6 and d 12 (Figure 4). Adipocyte protein 2 mRNA was also up-regulated ($P < 0.05$) on d 2 in response to differentiation, but lower ($P < 0.05$) on d 6 and 12 compared with d 2. Expression of PPARγ did not differ ($P > 0.05$) in d 2 and 6 cells compared with undifferentiated cells, but was up-regulated ($P < 0.05$) on d 12. There was an interaction of linoleic acid supplementation and day of harvest for SREBP, a lipogenic transcription factor. Gene expression of SREBP was decreased ($P < 0.05$) in unsupplemented cells on d 6 and d 12 compared with d 2, but was up-regulated ($P < 0.05$) in linoleic acid-treated cells on d 12 compared with d 2 (Figure 4). Other energy-intake and lipogenesis-

associated genes were not affected by linoleic acid supplementation. Both GLUT4 and FASN mRNA were up-regulated ($P < 0.05$) on d 12 compared with d 2 and undifferentiated cells.

Primary cell lines were confirmed to have a high proportion of preadipocytes through observation of morphological changes and staining of organized lipid droplets within cells, gene expression changes, and lipid profiles. Two days after differentiation media was applied, cultures demonstrated characteristic shape-change, from fusiform to spherical, and adipogenic gene expression through up-regulated adipocyte protein 2 (aP2) and SCD mRNA, which are markers for differentiation along with PPARγ (Chawla et al., 1994; Ohsaki et al., 2007). The master regulator of adipocyte regulation is classically stated as PPARγ; however, most of this work has been conducted in murine species (Ntambi and Kim, 2000). We did not observe marked up-regulation of PPARγ gene expression in this study. Similarly, Lengi and Corl (2010), when using the same hormonal induction of differentiation in primary bovine stromal vascular cultures, failed to detect up-regulated PPARγ gene expression. Despite a lack of PPARγ up-reguation in differentiated cultures, active lipogenesis was confirmed through increased labeled acetate incorporation into lipids (Lengi and Corl, 2010). Similarly, our cultures demonstrated lipid-filling, creation *de novo* lipid synthesis products, C16:0 and C18:0, and uptake exogenous linoleic acid. Additionally, in the absence of differentiation media, preadipocyte cultures had limited uptake of supplemented lipid (data not shown) suggesting our differentiation media was effective at inducing desired changes within the

cells. The responses we observed in our differentiated cultures were comparable with the literature (Aso et al., 1995; Ohsaki et al., 2007; Grant et al., 2008a).

Fatty acid profiles indicated that adipocytes absorbed linoleic acid from the media and began taking up and synthesizing fat within 6 d of applying the primary differentiation media. Increased fatty acids over time in unsupplemented cells suggests fatty acid sythethis, absorption from the media, or both was occurring. Reduced fatty acids over time due to linoleic acid supplementation could indicate a down-regualtion of lipogenesis, an up-regulation of β-oxidation, or both. Regulation of fatty acid synthesis can occur at many different levels. Each enzyme in the lipogenesis pathway can be regulated in addition to cellularity of adipose tissue. Fatty acid synthesis begins with the committed step of adding a carboxyl group to acetyl-CoA, forming malonyl-CoA, by ACC, which can be regulated allosterically and by phosphorylation. Fatty acid synthase is a large, multimeric protein that sequentially adds acetate to malonyl-CoA or a fatty acyl-CoA until the fatty acid reaches a length of 16 carbons, C16:0. It is primarily regulated through transcription. Up-regulation of FASN mRNA in differentiated cultures suggests lipogenic gene expression does not change until d 12; however, micrograms of fatty acids were increased by d 6. Despite increasing C16:0 over time in linoleic acid-treated cultures, there was no difference in gene expression of FASN compared with unsupplemented cells. Therefore, from this data, it is unclear whether linoleic acid affects fatty acid synthesis. In addition, biosynthesis pathways may be regulated at a level other than gene transcription. In fact, C16:1, C18:0 and C18:1 *cis*-9 all decreased over time in linoleic acid-treated cells compared with control cells suggesting an anti-

lipogenic effect of linoleic acid supplementation or an increase in β-oxidation. Though not significant, lipogenic gene and protein expression were numerically greater in bovine adipocytes treated with 50 μM linoleic acid on d 8 compared with non-fatty acid added controls (Lengi and Corl, 2010). Fatty acid data was not reported by Lengi and Corl (2010), so comparison of lipid profiles was not possible.

Stearoyl-CoA desaturase is the rate-limiting enzyme in the production of MUFA from SFA. The preferred substrates for SCD are C16:0 and C18:0, which become C16:1 and C18:1 *cis*-9, respectively. Because of its role in biosynthesis of MUFA, SCD can impact membrane fluidity and composition of stored triglycerides by changing the ratio of MUFA/SFA. Previously, fatty acid indices of MUFA/SFA have been correlated to SCD mRNA expression (Peter et al., 2009). Even though SCD gene expression was not affected by linoleic acid supplementation in Experiment 2, desaturase activity appeared to decrease in linoleic acid-treated cells based on a reduction in the C18:1 *cis*-9/C18:0 ratio. In unsupplemented cells, C16:1/C16:0 ratio did not change over time, but the C18:1 *cis*-9/C18:0 ratio increased over time, suggesting C18:0 may be a preferential substrate for desaturation compared with C16:0 in bovine adipocytes cultures or that synthesis rates of C16:0 closely mimic desaturation rates.

As reviewed by Ntambi and Miyazaki (2004), polyunsaturated fatty acids (PUFA) decrease SCD expression in murine cells. In bovine adipose tissue, however, increasing linoleic acid from the diet is associated with up-regulation of SCD mRNA in subcutaneous adipose tissue (Duckett et al., 2009). Transcription of SCD mRNA is thought to be controlled primarily by SREBP that binds to sterol response element in the

promoter region of the SCD gene, but it is also regulated by many other factors (Eberlé et al., 2004; Lay et al., 2002). We suspect timing and level of fatty acid supplementation to have a key role in linoleic acid's influence on SCD expression in bovine adipocyte cultures. Overall, expression of SCD mRNA was up-regulated prior to changes in fatty acid desaturation indices. However, desaturation indices are not always good indicator of SCD activity (Archibeque et al., 2005). This could indicate a lag in the translation of functional SCD1 protein or an additional level of regulation, microRNA for instance.

In conclusion, fatty acid composition changes with respect to time following induction of differentiation and also reflects changes in fatty acid composition of the media. Insulin supplemented at the current dose was not effective at altering lipogenic gene transcription or fatty acid profiles of bovine adipocyte cultures. Supplementing culture media with linoleic acid reduced SCD1 transcription at d 18 and decreased desaturation indices at d 6, 12, and 18. Bovine adipocyte cultures actively desaturate fatty acids, which leads to changes in fatty acid composition of the cultures. In addition, by d 6, fatty acid profiles of adipocyte cultures are sensitive to lipogenic regulation by exogenous fatty acid administration. Based on our data of fatty acid uptake and mRNA expression changes of differentiation-associated proteins, we propose to time future investigations of bovine adipocytes more closely with differentiation.

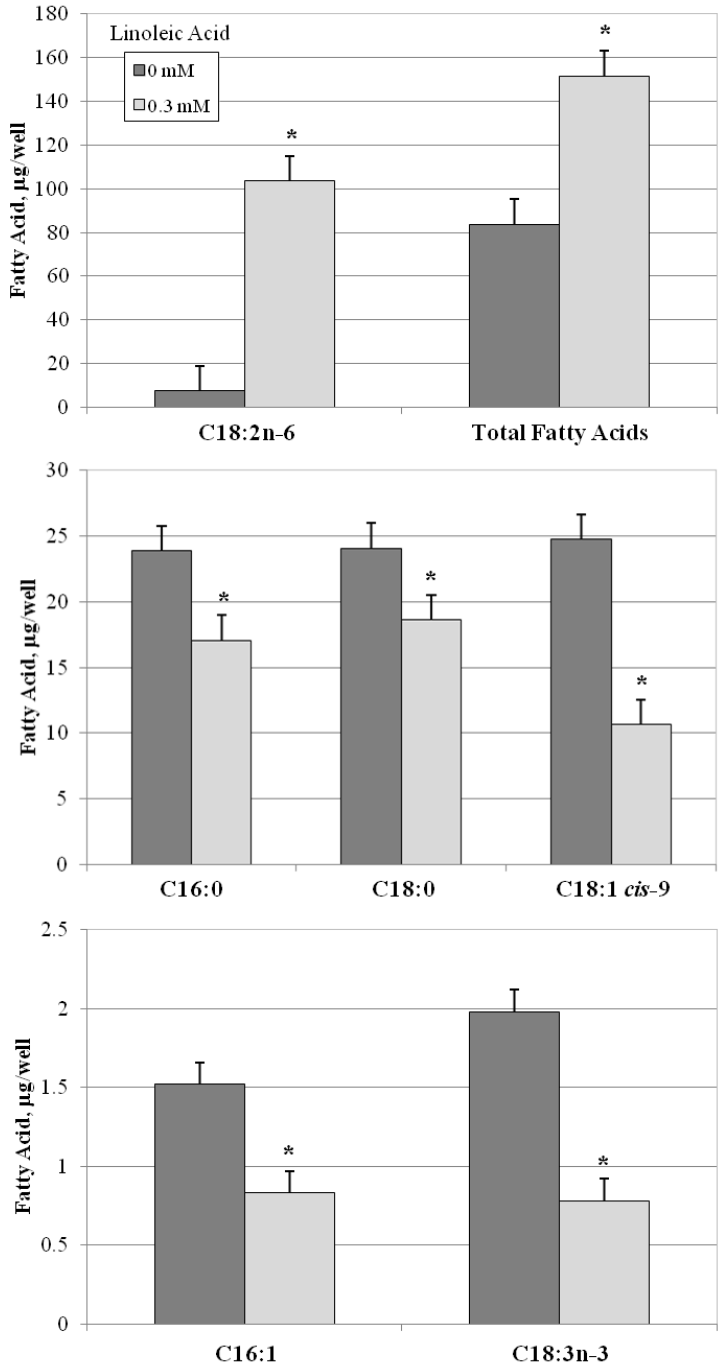

Figure 1. Main effect of linoleic acid supplementation (0 or 0.3 mM C18:2 n-6) on bovine adipocyte cultures on d 19. *$P < 0.05$, Means within a fatty acid differ.

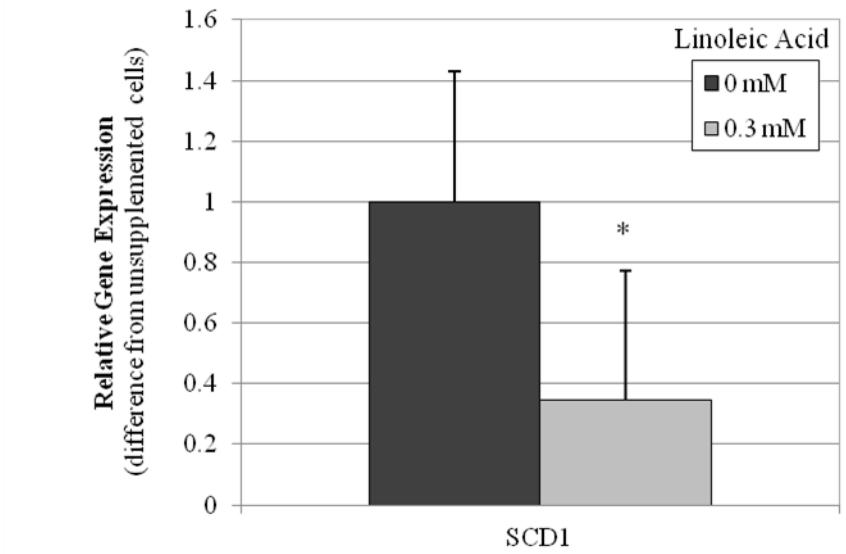

Figure 2. Main effect of linoleic acid supplementation (0 or 0.3 mM C18:2 n-6) on stearoyl-CoA desaturase 1 (SCD1) mRNA expression in bovine adipocyte cultures on d 19. *$P < 0.05$, Means differ.

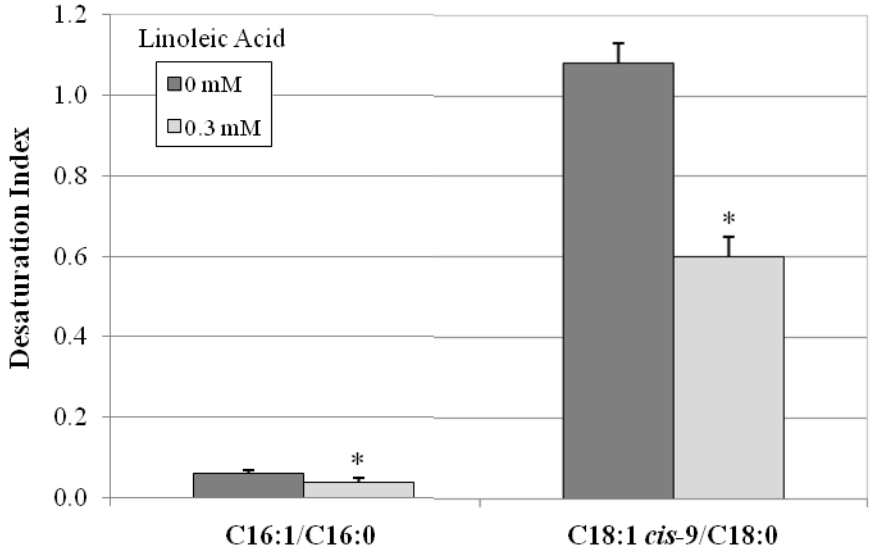

Figure 3. Desaturation indices of bovine adipocytes treated with 0 or 0.3 mM linoleic acid. *$P < 0.01$, means within a ratio differ.

Table 1. Gravimetric fatty acid composition of bovine adipocytes supplemented with 0 or 0.3 mM linoleic acid (C18:2 n-6) over time.

Fatty acid, μg/well	0 mM Linoleic Acid			0.3 mM Linoleic Acid			SEM	*p*-value		
	d 2	d 6	d 12	d 2	d 6	d 12		Linoleic Acid	Day	Interaction
C16:0	5.46^{d}	12.04^{c}	24.87^{a}	5.46^{d}	15.92^{b}	23.87^{a}	0.93	0.230	< 0.001	0.046
C16:1	0.34^{c}	0.86^{b}	1.98^{a}	0.34^{c}	0.64^{bc}	0.24^{c}	0.15	< 0.001	< 0.001	< 0.001
C18:0	8.01^{c}	12.12^{b}	22.73^{a}	8.01^{c}	11.56^{b}	13.03^{b}	0.97	< 0.001	< 0.001	< 0.001
C18:1 *cis*-9	5.36^{c}	14.34^{b}	33.53^{a}	5.36^{c}	6.30^{c}	5.29^{c}	0.71	< 0.001	< 0.001	< 0.001
C18:2 n-6	3.70^{c}	5.87^{c}	11.56^{c}	3.70^{c}	71.48^{b}	99.08^{a}	4.26	< 0.001	< 0.001	<0.0001
C18:3 n-3	ND^{c}	0.59^{b}	1.54^{a}	ND^{c}	0.02^{c}	ND^{c}	0.18	< 0.001	0.002	0.002
Total fatty acids	63.93^{c}	125.51^{bc}	163.30^{ab}	63.93^{c}	247.05^{a}	223.97^{ab}	26.8	0.027	0.130	0.432
C16:1/C16:0	0.066^{ab}	0.071^{ab}	0.08^{a}	0.066^{ab}	0.041^{ab}	0.010^{b}	0.02	0.046	0.819	0.424
C18:1 *cis*-9/C18:0	0.76^{c}	1.21^{b}	1.48^{a}	0.76^{c}	0.56^{d}	0.41^{d}	0.07	< 0.001	< 0.001	0.013
MUFA/SFA	0.44^{c}	0.63^{b}	0.75^{a}	0.44^{c}	0.25^{d}	0.15^{e}	0.03	< 0.001	< 0.001	< 0.001

$^{a\text{-}d}$ Within an entire row, means without a common superscript differ ($P < 0.05$)

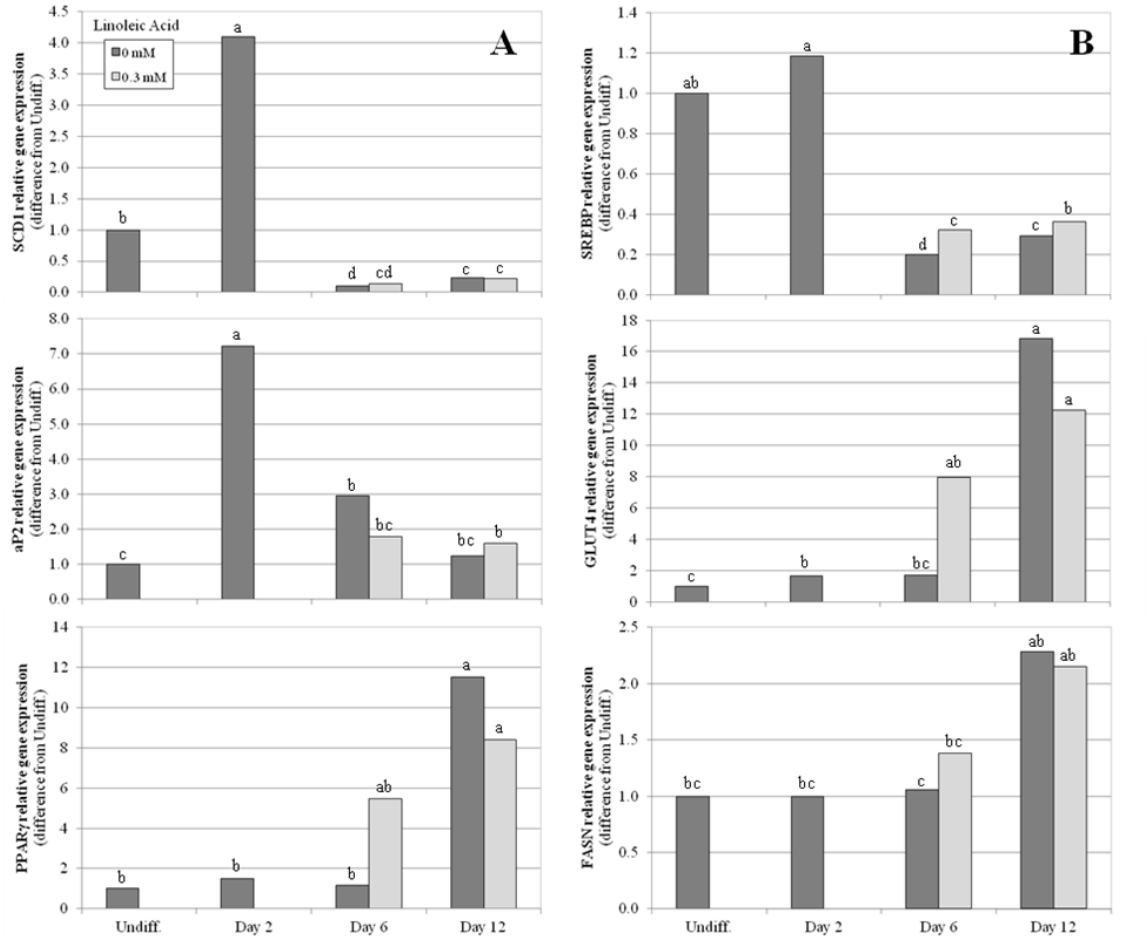

Figure 4. Real-time qRT-PCR data of bovine adipocytes cultured to d 2, 6, or 12 with 0 mM or 0.3 mM linoleic acid (C18:2 n-6). A. Expression of differentiation-associated and transcription factor mRNA [stearoyl-CoA desaturase 1 (SCD1), adipocyte protein 2 (aP2), and peroxisome proliferator-activated receptor γ (PPARγ)]. B. Expression of energy intake-associated and lipogenic mRNA [sterol regulatory element binding protein (SREBP), glucose transporter 4 (GLUT4), fatty acid synthase (FASN)]. C_T values were normalized to glyceraldehydes-3-phosphate dehydrogenase and set to relative expression of undifferentiated cultures (Undiff) on d 2. $^{a\text{-}d}$Bars without a common letter differ ($P < 0.05$).

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CHAPTER 3: SUPPLEMENTAL PALMITOLEIC ACID (C16:1 *cis*-9) REDUCES LIPOGENESIS AND DESATURATION IN BOVINE ADIPOCYTE CULTURES

ABSTRACT

Our objective was to determine if palmitoleic (C16:1 *cis*-9) acid supplementation to primary bovine adipocytes regulates lipogenic gene expression and rates of lipogenesis. Stromal vascular cells were isolated from 3 cattle, propagated, and frozen for use in this study. Cells were passaged 4 times, allowed to reach confluence, held for 2 d, and then differentiated with a standard hormone cocktail (d 0). At d 2, secondary differentiation media containing 1 of 4 levels of palmitoleic acid (0, 50, 150, or 300 μM) was added for 10 d. Cells were harvested on d 6 and 12 to assess fatty acid levels and gene expression. In addition, ^{13}C2 and ^{13}C18:0 stable isotopes were added on d 6 to measure lipogenesis and desaturase activity, respectively. Levels of C16:1 and total fatty acids increased ($P < 0.05$) linearly in response to palmitoleic acid supplement. Levels of C18:1 *cis*-11 and C20:1 *cis*-13 also increased ($P < 0.01$) in response to supplementation suggesting elongation of palmitoleic acid *in vitro*. Levels of C16:1, C18:1 *cis*-11 and total fatty acids were also greater ($P < 0.05$) at d 12 compared to d 6. In contrast, C16:0, C18:0, and C18:1 *cis*-9 levels decreased ($P < 0.05$) in response to palmitoleic acid supplementation and were not affected ($P > 0.05$) by harvest day. The ratio of C18:1 *cis*-9/C18:0 and fractional synthetic rate (FSR) of desaturation decreased ($P < 0.05$) in response to increasing palmitoleic acid supplementation. In addition, FSR of lipogenesis was reduced ($P < 0.05$) in palmitoleic acid-treated cells. Messenger RNA levels as

determined by qRT-PCR for stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FASN), and elongase protein 6 (ELOVL6) genes were reduced ($P < 0.05$) by palmitoleic acid supplementation. Expression of a β-oxidation gene, carnitine palmitoyltransferase 1A (CPT1A), was up-regulated ($P < 0.05$) with palmitoleic acid supplementation in a dose response manner. Supplementation of palmitoleic acid to bovine adipocytes results in increased incorporation of this fatty acid and its elongation products into the adipocyte, which down-regulates SCD1, FASN, and ELOVL6 to decrease lipogenesis and desaturation and up-regulates CPT1A to increase β-oxidation. These results suggest that palmitoleic acid, an end-product of desaturation, can act as a regulator of lipogenesis, desaturation, and β-oxidation in bovine adipocytes.

INTRODUCTION

Over the past decade, researchers have begun to view white adipose tissue, not as an inert energy storage site, but as a complex, metabolically active tissue (McGillis, 2005). Excess adipose tissue is not advantageous to human health, with regard to common medical disorders associated with obesity, or to livestock producers, who strive for a balance between maximal intramuscular fat and minimal subcutaneous fat which influence beef quality and yield grades, respectively. In addition, interest in fatty acid composition of meat and milk products has increased with concerns of saturated fatty acids (SFA) in the human diet. Research shows that adipocytes in white adipose tissue produce and secrete leptin (Flier, 1998), adipokines (MacDougald and Burant, 2007), and lipokines (Cao et al., 2008) that coordinate systemic metabolism.

Cao et al. (2008) identified palmitoleic (C16:1 *cis*-9) acid as a lipokine that increases insulin sensitivity in skeletal muscle and decreases lipogenesis in liver of mice. In this study, exogenous administration of palmitoleic acid to mice reduced lipogenic gene expression in the liver and increased insulin signaling in skeletal muscle. In humans, circulating palmitoleic acid level is strongly associated with abdominal obesity in children (Okada et al., 2005) and adults (Gong et al., 2010), and predictor of insulin sensitivity in adults (Stefan et al., 2010). In finishing beef cattle, the correlation between tissue palmitoleic and oleic (C18:1 *cis*-9) acid concentrations and carcass fat content are high (r = 0.68 and 0.81, respectively; Duckett unpublished data). In rodents and non-obese humans, the liver serves as the primary site of *de novo* lipogenesis; conversely, adipose tissue serves as the primary site of *de novo* lipogenesis in ruminants (Vernon,

1980; Smith and Crouse, 1984). Palmitoleic acid in circulation and adipose tissues originates predominately from *de novo* fatty acid synthesis as dietary sources of C16:1 are minor. Therefore, we hypothesize that palmitoleic acid supplementation to bovine adipocytes will decrease lipogenic gene expression and lipogenic rates *in vitro*.

MATERIALS AND METHODS

Cell Culture. Primary bovine stromal vascular (SV) cultures were harvested from adipose tissue of 18 mo old Angus crossbred cattle as described by Pratt et al. (2010) using slight modifications of methods described by Hirai et al. (2007). Cells were plated at 1 x 10^4 cells/cm^2 and passaged every 2 to 4 d when 60% confluent. Cells were incubated at 37°C under 5% CO_2 humidified atmosphere with media [Dulbecco's modified eagles medium (DMEM) containing 10% fetal calf serum (FCS), and 2X antibiotic/antimycotic (AB/AM; containing 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B)] replacement every 2 d. After 4 passages, cell lines were stored in liquid nitrogen at 1 x 10^6 cells/mL in freezing media (DMEM, 20% FCS, and 10% dimethyl sulfoxide) for later use.

Treatments. Individual cultures from 3 animals were used in duplicate for this study. Cells were thawed, passaged 3 times, and seeded in 6-well plates (1.6 cm^2/well) at 1 x 10^5 cells/cm^2. Cells were allowed to reach confluence, held for 2 d, and differentiated on d 0 with DMEM containing 5% FCS, 2X AB/AM, 2.5 μg/ml insulin, 0.5 mM 2-isobutyl-1-methylaxanthine (IBMX), 0.25 μM dexamethasone (DEX), 5 μM troglitazone (TRO), and 10 mM acetate (Hirai et al., 2007; Pratt et al., 2010). Secondary differentiation media (DMEM, 5% FCS, 2X AB/AM, 2.5 μg/mL insulin, 5 μM TRO, and 10 mM acetate) was applied for 4 (d 6) or 10 d (d 12) along with palmitoleic acid (C16:1 *cis*-9) at varying levels (0, 50, 150, or 300 μM). Palmitoleic acid was bound to bovine serum albumin (2:1, w/w) according to Van Harken et al. (1969) prior to media addition.

Cells were harvested on d 6 and d 12 for fatty acid composition and lipogenic gene expression. Before trypsin was applied, media was removed and cells were rinsed 3 times with PBS.

The differentiation hormones and media used in our experiment have been tested for their efficacy in inducing differentiation in bovine SV cultures (Aso et al., 1995; Grant et al., 2008a and b; Lengi and Corl, 2010). Classically, preadipocytes change shape from fibroblastic to round and accumulate lipid (Napolitano, 1963), in addition to changes in gene expression (Taniguchi et al., 2008). Previously, our SV cultures displayed characteristic changes in morphology, lipid-filling, and adipogenic gene expression upon hormonal treatment, consistent with adipocyte differentiation (Pratt et al., 2010). In the current study, cells changed shape from fibroblastic to round following differentiation and became lipid-filled with fatty acid supplementation (Figure 1).

Fatty acids. Cellular fatty acids were extracted and transmethylated as described by Folch et al. (1957) and Park and Goins (1994), respectively. Fatty acid methyl esters (FAME) were analyzed using an Agilent 6850 gas chromatograph (GC) equipped with an Agilent 7673A automatic sampler (Agilent Technologies, Inc., Santa Clara, CA). Separations were accomplished using a 100-m Supelco SP-2560 (Supelco, Inc., Bellefonte, PA) capillary column (0.25 mm i.d. and 0.20 μm film thickness) according to Duckett et al. (2002). Individual fatty acids were identified by comparison of retention times with standards (Sigma, St. Louis, MO; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating an internal standard, methyl tricosanoic (C23:0) acid,

into each sample during methylation and expressed as a weight percentage of total fatty acids per well.

Lipogenesis. To measure lipogenesis *in vitro*, we added stable isotope labeled acetate (2.5 mM 1-^{13}C2) to cultures treated with 0, 150, and 300 μM C16:1 on d 6 for 0, 6, 12, and 24 h. At harvest, cells were placed directly into 2:1 chloroform:methanol (vol/vol) to terminate all enzymatic activity. Fatty acid methyl esters were prepared as stated above for analysis with an Agilent 6890N GC equipped with an Agilent 5973 mass spectrometer (MS) using a 100-m Varian CP7489 (Varian Instruments Inc., Walnut Creek, CA) capillary column (0.25 mm i.d. and 0.20 μm film thickness). Samples were run in the chemical ionization mode with He as the carrier gas and CH_4 as the reagent gas. Ions of mass-to-charge ratio (*m*/*z*) 270 (*m*), 271 (*m* + 1), and 272 (*m* + 2) were selectively measured to calculate the isotopic enrichments of C16:0.

Enrichment of a sample treated with stable isotope can be calculated by several different methods. Because we were interested in calculating lipogenic rate in terms of the formation of a fatty acid polymer, C16:0, from repeating units of acetate, we used Mass Isotopomer Distribution Analysis (MIDA), which is ideal in this application (Wolfe and Chinkes, 2005). In essence, MIDA estimates the precursor enrichment pool ($MPE_{precursor}$) based on probabilities and uses it to calculate a fractional synthetic rate (FSR) of the product. We calculated the relative abundance (RA) of singly (*m* + 1) and doubly (*m* + 2) labeled C16:0 to unlabeled (*m*) C16:0 isotopomers. To account for background noise and natural abundance of ^{13}C isotope, the tracer-to-tracee ratio (TTR) was calculated as $TTR_{C16:0} = (RA_{sample} - RA_{blank}) * (1 - A)^n$, where A was the natural

abundance of ^{13}C, A = 0.011, and n was the number of C in the fatty acid molecule, n = 16 (Wolfe and Chinkes, 2005). Samples that were not exposed to labeled isotope served as 'blanks'. Precursor enrichment in molar percent excess (MPE) was estimated by:

$$MPE_{precursor} = \frac{2 \times (TTR_{(m+2)} / TTR_{(m+1)})}{(p-1) + (2 \times (TTR_{(m+2)} / TTR_{(m+1)})}$$

where, p is the number of precursor monomers present in the polymer, p = 8. Finally, our estimate of lipogenesis could be derived by calculating FSR (Wolfe and Chinkes, 2005).

$$FSR_{lipogenesis} = \frac{TTR_{C16:0,\ time1} - TTR_{C16:0,\ time0}}{(p \times MPE_{precursor})\ (1 - MPE_{precursor})^{p-1}\ (time1 - time0)}$$

Desaturation. Stearoyl-CoA desaturase 1 (SCD1) is the enzyme responsible for creating a double bond at the Δ^9 position of several fatty-acyl CoA and its preferred substrates are C16:0 and C18:0 (Enoch et al., 1976). Stearoyl-CoA desaturase transcription and activity can regulated by certain fatty acids (Ntambi, 1995), but the effect of palmitoleic acid supplementation on SCD1 activity has not been reported to date. Traditionally, western blotting is used to detect protein abundance; however, we have not been successful in blotting for bovine SCD1 using commercially available antibodies designed for human and mouse. Therefore, we focused our efforts on *in vitro* measurement of SCD1 activity using stable isotopes. Initially, we added 20 μM 1-^{13}C18:0 to cultures on d 6 as described above. Enrichment of C18:0 was detected by 6 h post-inclusion of tracer; however, TTR of C18:1 *cis*-9 was not evident in cultures (data not shown). Therefore, we repeated the experiment with 2 cell lines treated with 0 or 150

μM C16:1 and increased the amount of tracer to 100 μM 1-^{13}C18:0 on d 6. Samples were prepared and analyzed as described above for analysis with GC-MS. Ions of mass-to-charge ratio (*m*/*z*) 298 (*m*) and 299 (*m* + 1) were selectively measured to calculate the isotopic enrichments of C18:0. Similarly, isotope abundance of 296 (*m*) and 297 (*m* + 1) was measured for C18:1 *cis*-9. Since tracer enrichment can be measured in this case and the product of the SCD reaction is not a polymer of the tracer, it was not necessary to use MIDA. The FSR was calculated for rate of desaturation using the precursor enrichment, $MPE_{C18:0}$.

$$FSR_{desaturation} = \frac{TTR_{C18:1\ cis\text{-}9,\ time1} - TTR_{C18:1\ cis\text{-}9,\ time0}}{MPE_{C18:0} \times (time1 - time0)}$$

Gene Expression. Total cellular RNA was isolated from cells using the *mir*Vana microRNA Isolation kit (Ambion, Austin, TX) according to manufacturer's instructions and RNA quality as described by Duckett et al. (2009). Quality was assessed using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and Agilent Bioanalyzer 2100. All tcRNA samples used in real-time PCR (qRT-PCR) had a 260:280 absorbance ratio > 1.8 on the Nanodrop and RNA integrity number > 8.0 (1.0 to 10.0 scale) using Agilent RNA 6000 Nano kit. Real-time qRT-PCR was conducted using an Eppendorf MasterCycler ep realplex (Westbury, NY) with the QuantiTect SYBR Green RT-PCR One Step Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Two genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, were evaluated as housekeeping genes for data normalization (Duckett et al., 2009). To determine the appropriate housekeeping gene to be used to normalize the data, the

cycle threshold values (C_T) for GAPDH, β-actin, and all target genes per sample were entered into the BESTKEEPER program (http://www.gene-quantification.info). The program determines the most stable housekeeping gene to be used for normalization by repeated pair-wise correlation and regression analysis (Pfaffl et al., 2002). Both GAPDH and β-actin exhibited a correlation coefficient of 0.99 ($P < 0.001$) in the analysis and were suitable for data normalization. Primers for bovine mRNAs were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Primer sets were first evaluated according to Duckett et al. (2009) to verify identity. Genes of interest for this study were those involved in fatty acid biosynthesis including acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), fatty acid elongase (ELOVL) -5 and -6, SCD1, sterol regulatory element binding protein-1c (SREBP), and carnitine palmitoyl-transferase 1A (CPT1A).

Statistical Analysis. The data were analyzed using Proc GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC) for treatment comparisons over time. A two-way analysis of variance (ANOVA) was performed as a completely randomized design with factors fatty acid level (0, 50, 150, and 300 μM C16:1), day (d 6 and d 12) or time (0, 6, 12, and 24 h), and two-way interaction. Least squares means were computed and separated statistically using Fisher's Protected LSD test. In addition, linear regression was performed using the bivariate fit model of JMP Version 7 (SAS Institute Inc.). Relative gene expression data was analyzed using Pair-wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002) with preplanned treatment comparisons.

RESULTS AND DISCUSSION

Fatty acids. We successfully incorporated our fatty acid supplement into bovine adipocyte cultures based on detection of increasing amounts of C16:1 in palmitoleic acid-treated cells after all media was removed (Figure 2). Rates of palmitoleic acid incorporation into the adipocytes were 0.24 and 0.61 μg/well per μM supplemented palmitoleic acid for d 6 and 12 respectively. After completion of the fatty acid analysis, we discovered high concentrations of fatty acids believed to be C18:1 *cis*-11 and C20:1 *cis*-13. In order to confirm identity, ion masses were checked with GC-MS and samples were spiked with standards of known identity. The identity of these peaks were confirmed as being C18:1 *cis*-11 and C20:1 *cis*-13. A significant interaction ($P < 0.001$) between level of palmitoleic acid supplementation and day of harvest was present for total fatty acids, C16:1, C18:1 *cis*-11, and C20:1 *cis*-13 (Figure 2). Each of these fatty acids gravimetrically increased ($P < 0.01$) in response to higher levels of palmitoleic acid supplementation and the magnitude of response was greater ($P < 0.01$) on d 12 than d 6. No other fatty acid peaks detected showed a similar pattern of response ($P > 0.05$). As C18:1 *cis*-11 and C20:1 *cis*-13 were not present in high concentrations in control cells and they increased dramatically in cultures supplemented with palmitoleic acid, we suspect they were elongated from palmitoleic acid.

After accounting for any endogenous levels of C16:1, C18:1 *cis*-11, and C20:1 *cis*-13 in our cultures, we estimated the percentage of supplemental palmitoleic acid that was elongated. In the 50 μM palmitoleic acid-treated cells on d 6, 66 ± 2% of palmitoleic acid was elongated to C18:1 *cis*-11 and C20:1 *cis*-13. With higher levels of palmitoleic

acid supplementation, the percent of palmitoleic acid converted to C18:1 *cis*-11 and C20:1 *cis*-13 decreased ($P < 0.01$) to 55 ± 2% and 49 ± 2% for 150 and 300 μM treatment levels, respectively. Results were similar for d 12 cultures in that the percent of palmitoleic acid elongated was 69 ± 3%, 63 ± 2%, and 54 ± 2% for the 50, 150, and 300 μM palmitoleic acid treatment levels, respectively. Despite the linear incorporation of C16:1 into the adipocytes, the percentage elongated into C18:1 *cis*-11 and C20:1 *cis*-13 in bovine adipocytes was curvilinear suggesting that the elongase reaction was limited by another factor other than substrate availability.

There was also an interaction ($P < 0.05$) between level of palmitoleic acid supplementation and day of harvest for linoleic (C18:2 n-6) acid (Figure 3). On d 12, linoleic acid content did not differ ($P > 0.05$) with palmitoleic acid supplementation (Table 1). On d 6, linoleic acid content decreased ($P < 0.05$) with palmitoleic acid supplementation. Stearic (C18:0) acid content was lower ($P < 0.05$) at 50 and 300 μM compared to controls (0 μM). Oleic (C18:1 *cis*-9) and arachidonic (C20:4 n-6) acids were lower ($P < 0.05$) in palmitoleic acid supplemented cells, regardless of supplementation level. Palmitic (C16:0) acid content did not differ ($P > 0.05$) with palmitoleic acid supplementation.

Lipogenesis. We were able to detect enrichment of C16:0, calculated by TTR, in cells treated with 1-^{13}C2 in the media on d 6. There was a significant interaction ($P < 0.01$) of $TTR_{C16:0}$ between level of palmitoleic acid supplementation and time of labeled acetate incubation. All palmitoleic acid treatment levels had $TTR_{C16:0}$ greater than zero ($P < 0.01$) at 12 h, but cells treated with 150 μM palmitoleic acid had reduced ($P < 0.05$)

$TTR_{C16:0}$ compared with 0 μM palmitoleic acid-treated cells and numerically lower $TTR_{C16:0}$ than 300 μM palmitoleic acid-treated cells (Figure 4). At 24 h after 1-^{13}C2 treatment, $TTR_{C16:0}$ of 0, 150, and 300 μM cells differed ($P < 0.001$) from each other. Similarly, $FSR_{C16:0}$ from 0 to 24 h following labeled acetate treatment was reduced ($P < 0.05$) approximately 45 and 30% by 150 and 300 μM palmitoleic acid supplementation, respectively. Therefore, lipogenesis, as measured by the rate of labeled acetate incorporation into the primary product of *de novo* fatty acid synthesis, C16:0, was decreased by palmitoleic acid supplementation.

Overall, micrograms of C16:0 present in our cultures did not differ due to palmitoleic acid treatment, but synthesis of ^{13}C16:0 from labeled ^{13}C acetate was reduced in palmitoleic acid-treated cells. Unchanged micrograms of C16:0 could indicate that C16:0 was not desaturated or elongated, but was conserved as a SFA in palmitoleic acid-treated cells. The ratio of MUFA/SFA is a critical factor effecting membrane fluidity and survivability of a cell (Ntambi and Miyazaki, 2004).

Desaturation. The ratios of monounsaturated fatty acids (MUFA) to SFA are often calculated as indicators of desaturation and have been associated with SCD1 activity (Baumgard et al., 2002; Mosley and McGuire, 2007). Since our fatty acid treatment confounded the C16:1/C16:0 ratio and peaks for C14:1 and C14:0 were not detectable, we used C18:1 *cis*-9/C18:0 ratio as our desaturation index. There was no main effect of harvest day on C18:1 *cis*-9/C18:0 ratio, but level of palmitoleic acid supplementation reduced ($P < 0.001$) the C18:1 *cis*-9/C18:0 ratio (Table 2). The 50, 150, and 300 μM palmitoleic acid treatment groups had lower ($P < 0.05$) C18:1 *cis*-9/C18:0

ratio compared with 0 μM palmitoleic acid-supplemented cells. In addition, cells treated with 300 μM palmitoleic acid had reduced ($P < 0.05$) desaturation index compared with cells treated with 50 μM palmitoleic acid. These results suggest that desaturation was decreased by palmitoleic acid supplementation; however, desaturation ratios are not always reflective of SCD1 activity (Archibeque et al., 2005).

To get a more accurate measurement of desaturation activity than simply using a desaturation index, we measured desaturation of stable isotope tracer in our cultures. We were able to detect significant enrichment of C18:0 in our cells 6 h following 1-^{13}C18:0 addition. There was no effect of palmitoleic acid supplementation on $TTR_{C18:0}$, only a main effect of time. Tracer-to-tracee ratios of C18:0 at 6 (0.12 ± 0.02), 12 (0.12 ± 0.02), and 24 h (0.17 ± 0.02) time points following the addition of label were greater ($P < 0.001$) than $TTR_{C18:0}$ at 0 h (0.00 ± 0.02), but not different from each other. For $TTR_{C18:1\ cis\text{-}9}$, there was significant interaction ($P < 0.01$) between level of palmitoleic acid supplementation and time of incubation with 1-^{13}C18:0. Enrichment of C18:1 *cis*-9 was first detected at 12 h for 0 μM palmitoleic acid treated cells and at 24 h for 150 μM palmitoleic acid treated cells (Figure 4). At 24 h when enrichment was above background for both levels of palmitoleic acid, $TTR_{C18:1\ cis\text{-}9}$ was greater ($P < 0.001$) in 0 μM palmitoleic acid cells than in 150 μM palmitoleic acid-treated cells. Similarly, the desaturation rate, calculated as $FSR_{C18:1\ cis\text{-}9}$ from 0 to 24 h following the addition of 1-^{13}C18:0, was reduced by more than 70% in cells supplemented with palmitoleic acid.

Gene Expression. Fatty acid synthase mRNA was down-regulated ($P < 0.01$) by 35 and 25% in 50 and 150 μM palmitoleic acid-treated cells, respectively (Figure 5). At

300 μM palmitoleic acid, FASN mRNA expression was not different ($P > 0.05$) from control (0 μM). Gene expression of ACC was not different ($P < 0.05$) in palmitoleic acid-supplemented cells compared to controls. Both ACC and FASN are key enzymes in *de novo* fatty acid synthesis. Fatty acid synthesis begins with the committed step of adding a carboxyl group to acetyl-CoA, forming malonyl-CoA, by ACC, which can be regulated allosterically, by phosphorylation, and transcriptionally. Fatty acid synthase is a large, multimeric protein that sequentially adds acetate to malonyl-CoA or a fatty acyl-CoA until the fatty acid reaches a length of 16 carbons, C16:0. Changed in FASN gene expression with palmitoleic acid supplementation followed a similar pattern as rate of lipogenesis using $^{13}C2$; however, gene expression was unchanged at 300 μM but lipogenic rate was still reduced compared to controls.

Expression of SCD1 mRNA was down-regulated ($P < 0.05$) by 60% or more at all three levels of palmitoleic acid supplementation compared to control (0 μM). Stearoyl-CoA desaturase is the enzyme responsible for the desaturation of SFA to MUFA by inserting a double bond in the Δ^9 position. As reviewed by Ntambi and Miyazaki (2004), SCD1 has been extensively studied especially in the murine model. Transcription of SCD1 mRNA is controlled primarily by SREBP which binds to sterol response element in the promoter region of the SCD1 gene (Lay et al., 2002; Eberlé et al., 2004). The promoter region of the bovine SCD1 gene is contains a fat specific element, PUFA response element, and SREBP-response region (Keating et al., 2006). Expression of SCD1 is also regulated by its products and not by the availability of its substrates (Keating et al., 2006). Our study confirmed that palmitoleic acid, an end-product of

SCD1, also down-regulates SCD1 mRNA expression in bovine cells. In addition, the MUFA/SFA ratio and $FSR_{desaturase}$ show that palmitoleic acid impacts actual activity of the desaturase as well. Due to the similarities between the percent decrease in desaturase activity and gene expression, the regulation of palmitoleic acid on SCD1 most likely occurs at the level of transcription; although, Cao et al. (2008) report SCD1 protein stability may also be affected by palmitoleic acid. The PUFA response element in the bovine SCD1 promoter region is the most likely target of transcriptional repression by palmitoleic acid (Keating et al., 2006). In a mouse reporter assay, promoter activity of SCD1 was suppressed by palmitoleic acid supplementation (Cao et al., 2008). When the PUFA response element of the SCD1 promoter region was mutated, palmitoleic acid no longer suppressed SCD1 promoter activity (Cao et al., 2008).

Several elongase enzymes are present in mammalian cells and responsible for that add acetyl-CoA molecules to the carboxylic acid end of a fatty acid hydrocarbon skeleton. However, each elongase isoform has preferred fatty-acyl substrates of particular C length (Guillou et al., 2010). In humans, most ELOVL proteins identified elongate PUFA greater than or equal to 18C; however, ELOVL6 is attributed to the conversion of 12 to 16C SFA and MUFA, including C16:1 to C18:1 *cis*-11 (Matsuzaka et al., 2002; Leonard et al., 2004). It has been proposed that palmitoleic acid can be elongated into C18:1 *cis*-11 and potentially elongated further into a 20C fatty acid, C20:1 *cis*-13 (Matsuzaka and Shimano, 2009). In addition, ELOVL5 is reported to have some activity for this reaction (Wang et al., 2006), especially in absence of ELOVL6 (Matsuzaka et al., 2007). Primarily regulated by lipogenic transcription factors (Leonard

et al., 2004; Wang et al., 2006), these 2 ELOVL isoforms are the only bovine elongase enzymes published on NCBI to date (Zimin et al., 2009). As FASN is not capable of synthesizing fatty acids greater than 16C in length, acetate molecules were most likely added to C16:1 *cis*-9 by ELOVL6 in our study. Elongation with acetate increased the fatty acid chain length to 18 and 20C, subsequently changing the double bond to Δ^{11} and Δ^{13} positions, respectively, with each 2C addition.

In our adipocyte cultures, CPT1A mRNA was up-regulated ($P < 0.05$) by palmitoleic acid supplementation. Carnitine palmitoyl-transferase 1A is responsible for transporting long chain fatty acids through the outer mitochondrial membrane for the purpose of β-oxidation in the mitochondrial matrix. In ruminants, CPT1A is expressed in numerous tissues including adipose tissue in contrast to a more restricted tissue abundance in monogastrics (Price et al., 2003). Inhibition of CPT1A is primarily attributed to malonyl-CoA, the product of the ACC reaction, in monogastric species (McGarry and Brown, 1997). Price et al. (2003) found N-terminal sequence differences in ovine CPT1 that alter enzyme kinetics for certain substrates, which differs from that of the rat. Palmitoleic acid supplementation stimulates CPT1A transcription which may lead to increase β-oxidation and ATP production.

Fatty acid synthase, ELOVL6, and SCD1 genes are all transcriptionally controlled by SREBP (Eberlé et al., 2004), suggesting coordinated regulation. There was no effect ($P > 0.05$) of palmitoleic acid supplementation on SREBP gene expression (data not shown). However, post-translational modification is the primary level of regulation for SREBP activity, not transcriptional regulation (Brown and Goldstein, 1997). Therefore,

one would not expect mRNA expression of this transcription factor to be altered, even if the protein is regulating lipogenic genes.

Overall, supplementation of palmitoleic acid to bovine adipocytes results in increased incorporation of this fatty acid and its elongation products into the adipocyte, which down-regulates SCD1, FASN, and ELOVL6 to decrease lipogenesis and desaturation and up-regulates CPT1A to increase β-oxidation. These results suggest that exogenous palmitoleic acid, an end-product of desaturation, acts as a regulator of lipogenesis, desaturation, and β-oxidation in bovine adipocytes.

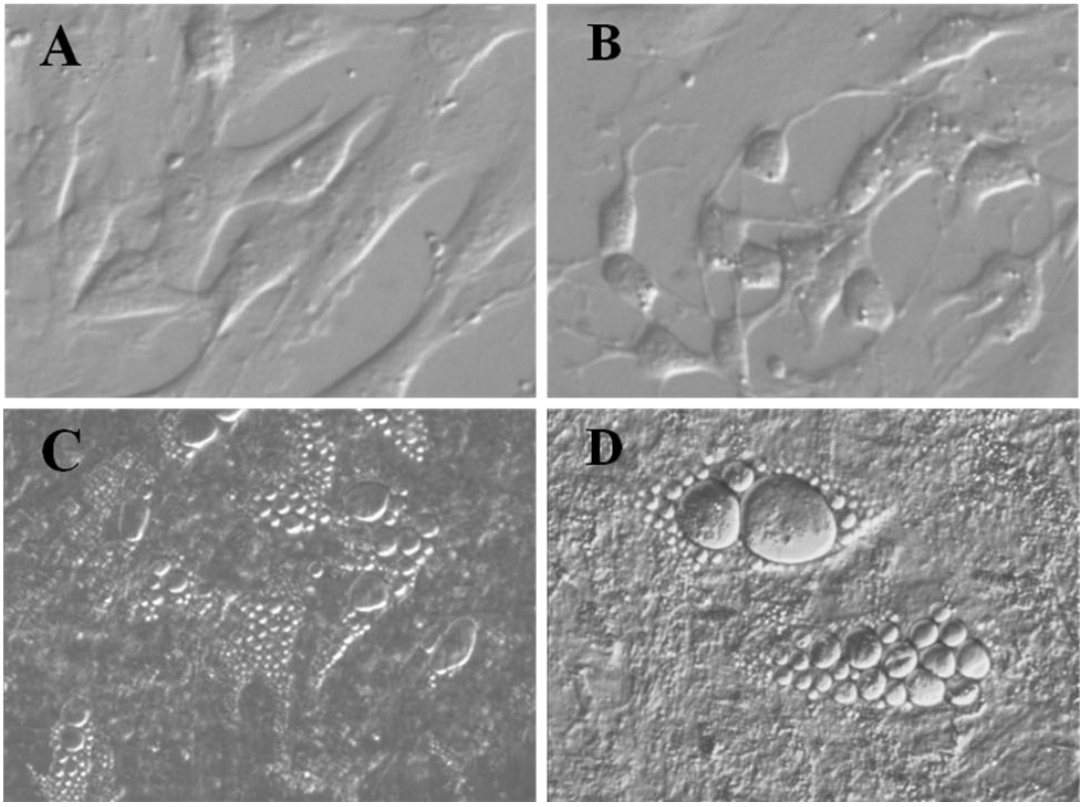

Figure 1. Nomarski interference contrast microscopy at 40X magnification showed morphological change in cells on d 2 and lipid filling in palmitoleic acid-supplemented cells on d 6 and d 12 post-differentiation. Undifferentiated preadipocytes on d 2 (A) were stellate in appearance and cells treated with differentiation media on d 0 were round in shape by d 2 (B). Cells treated with palmitoleic acid appeared to have greater lipid droplet size on d 6 (C) compared with d 12 (D).

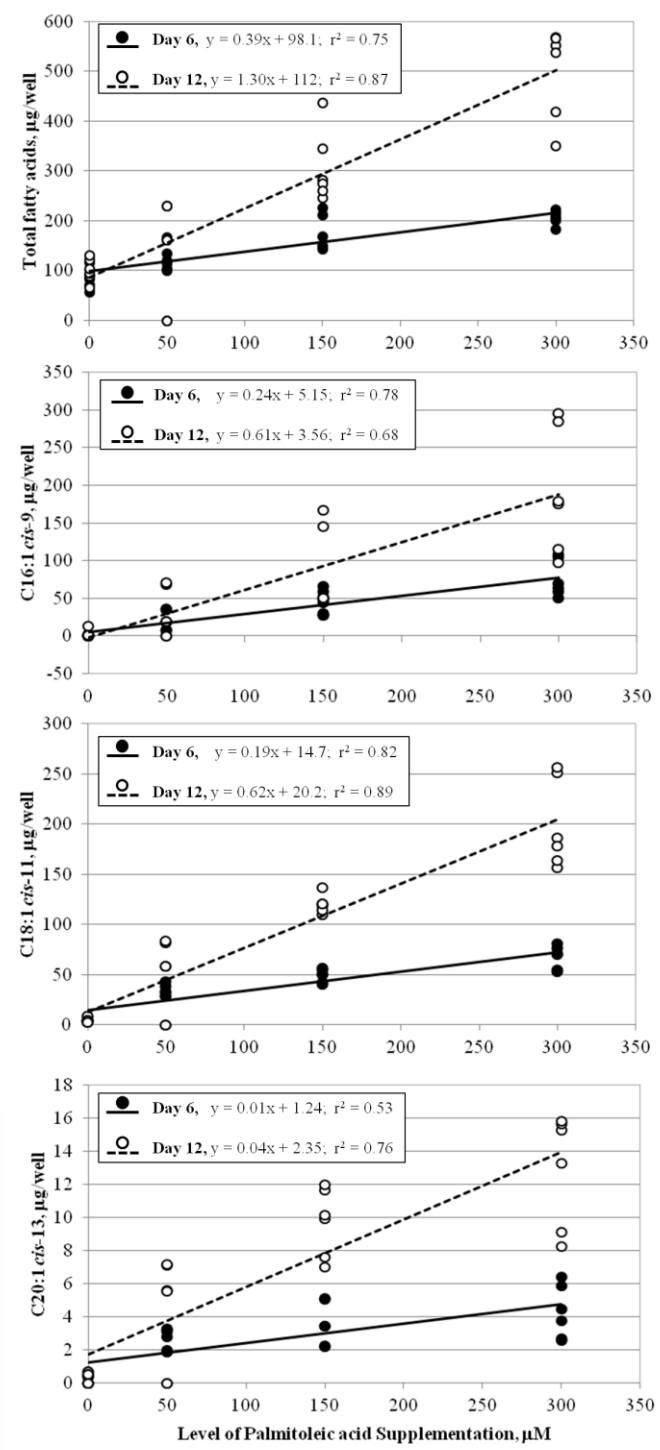

Figure 2. Scatterplot and linear regression of total fatty acids, palmitoleic (C16:1), *cis*-vaccenic (C18:1 *cis*-11), and eicosenoic (C20:1 *cis*-13) acids by level of palmitoleic acid supplementation. For each of these fatty acids, there was a significant interaction of palmitoleic acid supplementation level and day of harvest ($P < 0.001$).

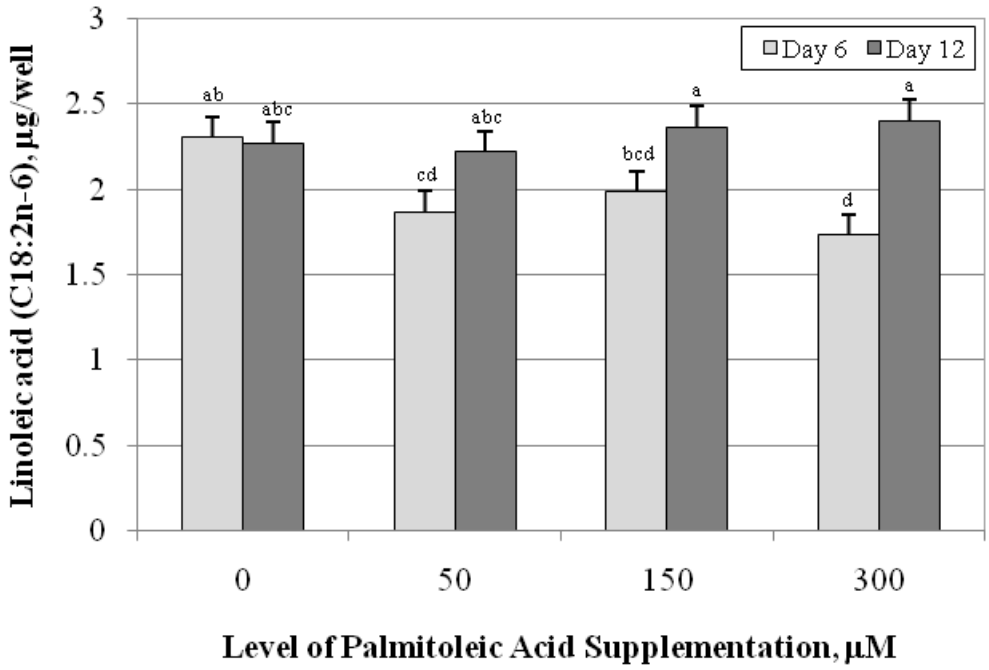

Figure 3. Linoleic acid (C18:2 n-6) in bovine adipocytes supplemented with 0, 50, 150, or 300 µM palmitoleic acid. An interaction between level of palmitoleic acid supplementation and day of harvest was present ($P < 0.05$). [a-d]Bars lacking a similar letter differ ($P < 0.05$).

Table 1. Main effect of level of palmitoleic acid supplementation on palmitic (C16:0), stearic (C18:0), oleic (C18:1 *cis*-9) acids, and desaturation index (C18:1 *cis*-9/C18:0) of bovine adipocytes.

Fatty acid, μg/well	Level of Palmitoleic Acid Supplementation				SEM	*p* -value*
	0 μM	50 μM	150 μM	300 μM		
C16:0	14.83	12.79	16.13	13.24	1.52	0.433
C18:0	15.41[a]	11.20[b]	13.45[ab]	10.84[b]	1.10	0.023
C18:1 *cis*-9	15.35[a]	9.42[b]	11.02[b]	7.34[b]	1.37	0.002
C20:4	6.57[a]	5.27[b]	5.10[b]	5.22[b]	0.19	< 0.001
C18:1 *cis*-9/C18:0	1.01[a]	0.84[b]	0.78[bc]	0.68[c]	0.04	< 0.001

[a-f] Within a row, means without a common superscript differ ($P < 0.05$)

[*] Probability of type I error with null hypothesis of all treatment means being equal.

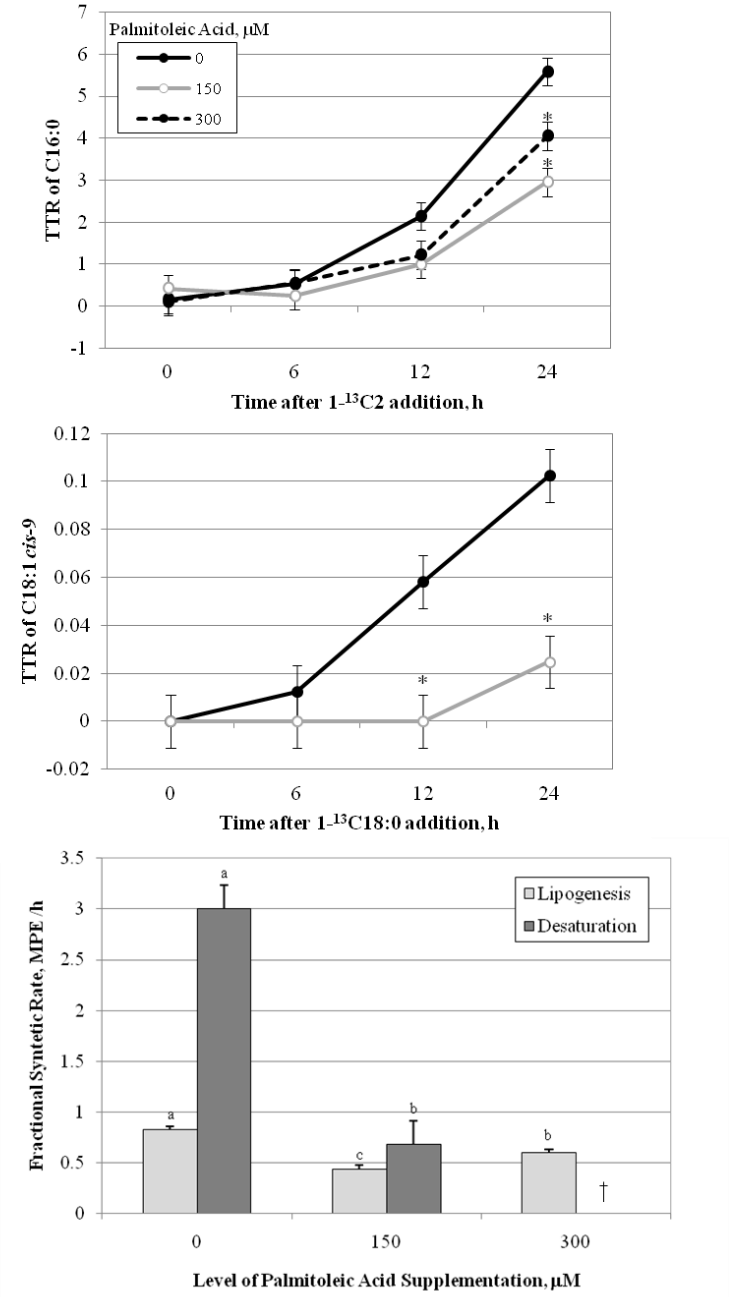

Figure 4. Tracer-to-tracee ratio (TTR) and fractional synthetic rate for the production of ^{13}C16:0 from 1-^{13}C2 (lipogenesis) and 1-^{13}C18:1 *cis*-9 from 1-^{13}C18:0 (desaturation) in bovine adipocyte cultures treated with palmitoleic acid, respectively. Molar percent excess (MPE) per h was calculated over a 24 h period following stable isotope addition to the media. *Within a time point, palmitoleic acid-treated cells differed from unsupplemented cells ($P < 0.05$). $^{a\text{-}c}$Bars of the same color without a similar letter differed ($P < 0.05$). † Analysis of 300 µM level was not performed for desaturation activity.

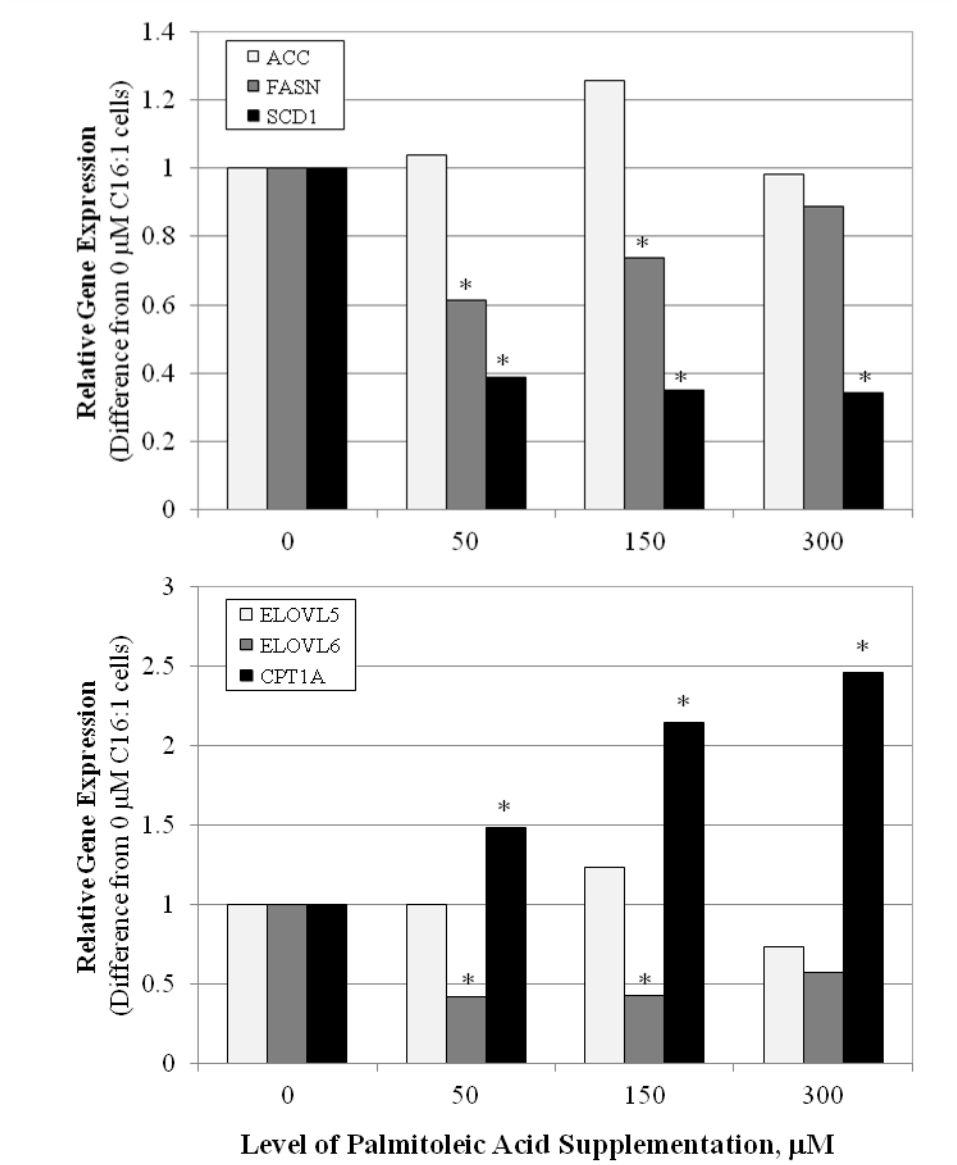

Figure 5. Relative expression of lipogenic genes in cells supplemented with 0, 50, 150, and 300 μM C16:1 to d 6 post-differentiation. Acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), elongation of long chain fatty acids protein (ELOVL) 5 and 6, and carnitine palmitoyl-transferase 1A (CPT1A). C_T a values were normalized to glyceraldehyde-3-phosphate dehydrogenase. *$P < 0.05$, Gene expression was up- or down-regulated compared with 0 μM palmitoleic acid-treated cells.

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CHAPTER 4: PALMITOLEIC (C16:1 *cis*-9) AND *CIS*-VACCENIC ACID (C18:1 *cis*-11) ALTER LIPOGENESIS IN BOVINE ADIPOCYTE CULTURES

ABSTRACT

Our objectives were to confirm elongation products palmitoleic acid (C16:1 *cis*-9) and determine if palmitoleic acid or its elongation products or both are responsible for decreased desaturation and lipogenesis rates previously observed in cultured bovine adipocytes supplemented with palmitoleic acid. Stromal vascular cells were isolated from 3 cattle, propagated, and frozen for use in this study. Cells were passaged 4 times, allowed to reach confluence, held for 2 d, and then differentiated with a standard hormone cocktail (d 0). At d 2, secondary differentiation media containing 1 of 4 levels of fatty acid [0 μM fatty acid (control), or 150 μM palmitic (C16:0), palmitoleic, or *cis*-vaccenic (C18:1 *cis*-11)] for 4 d. On d 6, cells were harvested for fatty acid analysis. In addition, cells were incubated with ^{13}C16:1, ^{13}C2, or ^{13}C18:0 on d 6 to estimate lipogenesis and desaturation rates and confirm elongation products of palmitoleic acid using gas chromatography-mass spectrometry. Incorporation of ^{13}C16:1 into cells and presence of ^{13}C label in C18:1 *cis*-11 confirmed it as an elongation product of palmitoleic acid in bovine adipocytes. Additionally, ^{13}C label was seen in C20:1 *cis*-13 and CLA *cis*-9, *cis*-11. The rate of ^{13}C16:1 elongation to ^{13}C18:1 *cis*-11 was greater ($P < 0.01$) in control cells compared with cell supplemented with palmitoleic acid prior to d 6. In addition, the elongation rate of control cells increased (P 0.05) over 36 h; whereas, elongation in palmitoleic acid-treated cells remained constant over the 36 h period. In

palmitoleic acid-supplemented cells, C16:1, C18:1 *cis*-11, and C20:1 *cis*-13 were elevated ($P < 0.05$) compared with control cells. In *cis*-vaccenic acid-supplemented cells, C18:1 *cis*-11 and C20:1 *cis*-13 were elevated ($P < 0.05$) compared with controls, but C16:1 was not. Synthesis of ^{13}C16:0 from ^{13}C2 was reduced ($P < 0.05$) in palmitoleic acid and *cis*-vaccenic acid-treated compared with control cells following 36 h incubation. The C18:1 *cis*-9/C18:0 ratio was decreased ($P < 0.05$) in palmitoleic acid-treated cells compared with all other treatments. By 12 h of ^{13}C18:0 incubation, cells supplemented with palmitoleic acid had reduced ($P < 0.05$) ^{13}C18:1 *cis*-9 compared with all other treatments. Therefore, palmitoleic acid is actively elongated *in* vitro and its elongation product, cis-vaccenic acid, can also reduce lipogenesis. However, inhibition of desaturation previously reported by our lab can be directly attributed to palmitoleic acid and not its elongation products, C18:1 *cis*-11 or, by extension, C20:1 *cis*-13.

INTRODUCTION

Excess adipose tissue can be detrimental to human health with regard to common medical disorders associated with obesity. In addition, excess adipose tissue accumulation in livestock animals is costly to producers as wasted feed resources and discounts or loss of premiums for carcass cutability. As components of both human and livestock diets, fatty acids have become a recent focus in energy metabolism research. In the public eye, interest in fatty acid composition of meat and milk products has increased in recent years with rising concerns of saturated fatty acids (SFA) in the human diet. Generally perceived, SFA are bad for human health; whereas, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are beneficial.

White adipose tissue is not longer considered an inert energy storage site, but a complex, metabolically active tissue (McGillis, 2005). The functional unit of white adipose tissue, the adipocyte, synthesizes and stores fatty acids and secretes hormones. Hormones produced by adipocytes include leptin (Flier, 1998), adipokines (MacDougald and Burant, 2007), and a newly, proposed class of hormone, "lipokine", in which fatty acids can act as signals to other body tissues (Cao et al., 2008). Whether as a component of human or livestock diets, palmitoleic acid may act to regulate metabolism. A lipidomic study by Cao et al. (2008) identified palmitoleic acid (C16:1 *cis*-9) as another fatty acid that may act a hormone. They reported palmitoleic acid functioned as a lipokine which increased insulin sensitivity in skeletal muscle and decreased lipogenesis in liver of mice. In contrast to murine animals, cattle synthesize fatty acids *de novo* from acetate, predominately in adipose tissue (Vernon, 1980; Smith and Crouse, 1984).

Similarly, we found palmitoleic acid functioned to reduce *de novo* fatty acid synthesis and reduce lipogenic gene expression in cultured bovine adipocytes (Burns et al., 2010). Supplementing bovine adipocyte cultures with palmitoleic acid resulted increased C16:1, cis-vaccenic (C18:1 *cis*-11) and eicosenoic acid (C20:1 *cis*-13). We hypothesize that C18:1 *cis*-11 and C20:1 *cis*-13 are being elongated from palmitoleic acid and could be responsible for the lipokine effects seen with palmitoleic acid supplementation in bovine adipocytes. Therefore, the objectives of this study were to trace palmitoleic acid elongation in bovine adipocytes and test lipogenesis and desaturation effects of C18:1 *cis*-11.

MATERIALS AND METHODS

Cell Culture. Primary bovine stromal vascular (SV) cultures were harvested from adipose tissue of 18 mo old Angus crossbred cattle as described by Pratt et al. (2010) using slight modifications of methods described by Hirai et al. (2007). Cells were plated at 1 x 10^4 cells/cm^2 and passaged every 2 to 4 d when 60% confluent. Cells were incubated at 37°C under 5% CO_2 humidified atmosphere with media [Dulbecco's modified eagles medium (DMEM) containing 10% fetal calf serum (FCS), and 2X antibiotic/antimycotic (AB/AM; containing 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B)] replacement every 2 d. After 4 passages, cell lines were stored in liquid nitrogen at 1 x 10^6 cells/mL in freezing media (DMEM, 20% FCS, and 10% dimethyl sulfoxide) for later use.

Treatments. Individual cultures from 2 animals were used in duplicate for each of these 2 experiments. Cells were thawed, passaged 3 times, and seeded in plates at 1 x 10^5 cells/cm^2. Cells were allowed to reach confluence, held for 2 d, and differentiated on d 0 with DMEM containing 5% FCS, 2X AB/AM, 2.5 μg/ml insulin, 0.5 mM 2-isobutyl-1-methylaxanthine (IBMX), 0.25 μM dexamethasone (DEX), 5 μM troglitazone (TRO), and 10 mM acetate (Hirai et al., 2007; Pratt et al., 2010). Secondary differentiation media (DMEM, 5% FCS, 2X AB/AM, 2.5 μg/mL insulin, 5 μM TRO, and 10 mM acetate) was applied for 4 (d 6) along with 0 μM additional fatty acids (control) or 150 μM fatty acids. According to the objectives of the experiment, cells were either harvested on d 6 for fatty acid composition and lipogenic gene expression or treated with

stable isotopes on d 6 for enzymatic activity assays. Previously, our stromal vascular cultures displayed characteristic changes in morphology, lipid-filling, and adipogenic gene expression upon hormonal treatment, consistent with adipocyte differentiation by d 6 (Pratt et al., 2010).

Experiment 1. Previously, our palmitoleic acid-treated cultures showed a dramatic increase in C18:1 *cis*-11 and C20:1 *cis*-13. It has been proposed that palmitoleic acid can be elongated into C18:1 *cis*-11 and potentially elongated further into a 20C fatty acid (Matsuzaka and Shimano, 2009). To confirm C18:1 *cis*-11 and C20:1 *cis*-13 as elongation products and to measure elongation *in vitro*, we added stable isotope labeled palmitoleic acid (150 μM U-^{13}C16:1) on d 6 for 0, 6, 12, 24, and 36 h to adipocytes cultures which had been treated until d 6 with 0 (control) or 150 μM palmitoleic acid. At harvest, cells were washed 3 times with PBS, removed with trypsin, and placed directly into 2:1 chloroform:methanol (vol/vol) to terminate all enzymatic activity. Cellular fatty acids were extracted using Folch et al. (1957) and transmethylated according to Park and Goins (1994). Fatty acid methyl esters were analyzed using an Agilent 6890N gas chromatograph (GC; Agilent Technologies, Inc., Santa Clara, CA) equipped with an Agilent 5973 mass spectrometer (MS) using a 100-m Varian CP7489 (Varian Instruments Inc., Walnut Creek, CA) capillary column (0.25 mm i.d. and 0.20 μm film thickness). Samples were run in the chemical ionization mode with He as the carrier gas and CH_4 as the reagent gas.

Ions of mass-to-charge ratio (*m*/*z*) 268 (*m*) and 284 (*m* + 16) were selectively measured to calculate the isotopic enrichments of C16:1. Similarly, ion abundance of

298 (*m*) and 312 (*m* + 16) were measured for C18:1 *cis*-11. We calculated the relative abundance (RA) of labeled (*m* + 16) to unlabeled (*m*) isotopomers of C16:1 and C18:1 *cis*-11. Fatty acid enrichment was calculated as tracer-to-tracee ratio (TTR), according to Wolfe and Chinkes (2005).

$$TTR_{sample} = (RA_{sample} - RA_{blank})$$

Samples that were not exposed to labeled isotope served as 'blanks'. Molar percent excess (MPE) of C16:1 was calculated by TTR/(TTR + 1) and fractional synthetic rate (FSR) of elongation was calculated from $TTR_{C18:1cis11}$ and $MPE_{C16:1}$.

$$FSR_{elongation} = \frac{TTR_{C18:1\ cis\text{-}11,\ time1} - TTR_{C18:1\ cis\text{-}11,\ time0}}{MPE_{C16:1} \times (time1 - time0)}$$

Experiment 2. The objective of this experiment was to determine if palmitoleic acid or an elongation product was responsible for changes in lipogenesis. Therefore, we treated adipocytes cultures with 0 μM fatty acids (control) or 150 μM palmitic (C16:0), palmitoleic, or *cis*-vaccenic acids on d 2 to d 6. Fatty acid methyl esters (FAME) were prepared from cultures on d 6 as stated above for analysis with an Agilent 6850 GC equipped with an Agilent 7673A automatic sampler. Separations were accomplished using a 100-m Supelco SP-2560 (Supelco, Inc., Bellefonte, PA) capillary column (0.25 mm i.d. and 0.20 μm film thickness) according to Duckett et al. (2002). Individual fatty acids were identified by comparison of retention times with standards (Sigma, St. Louis, MO; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating an internal standard, methyl tricosanoic (C23:0) acid, into each sample during methylation and expressed as a weight percentage of total fatty acids per well.

Lipogenesis. To measure lipogenesis *in vitro*, we substituted unlabeled acetate in the culture media on d 6 with stable isotope labeled acetate (10 mM 1-^{13}C2) to cultures treated with 0 μM fatty acids (control) or 150 μM palmitoleic or *cis*-vaccenic acid for 0, 12, 24, and 36 h. Cells were harvested and FAME prepared as described above. Ions of mass-to-charge ratio (*m*/*z*) 270 (*m*), 271 (*m* + 1), and 272 (*m* + 2) were selectively measured to calculate the isotopic enrichments of C16:0, the primary product of *de novo* lipogenesis.

The formation of a fatty acid polymer, C16:0, from repeating units of acetate is an ideal application of Mass Isotopomer Distribution Analysis (MIDA; Wolfe and Chinkes, 2005). Therefore, we used this approach to estimate enrichment in our precursor pool and calculate FSR of lipogenesis. First, we calculated RA of singly (*m* + 1) and doubly (*m* + 2) labeled C16:0 to unlabeled (*m*) C16:0 isotopomers. To account for background noise and natural abundance of ^{13}C isotope when using a singly-labeled tracer, the enrichment of C16:0 was calculated as $TTR_{C16:0} = (RA_{sample} - RA_{blank}) * (1 - A)^n$, where A was the natural abundance of ^{13}C, A = 0.011, and n was the number of C in the fatty acid molecule, n = 16 (Wolfe and Chinkes, 2005). Samples that were not exposed to labeled isotope served as 'blanks'. Precursor enrichment in molar percent excess (MPE) was estimated by:

$$MPE_{precursor} = \frac{2 \times (TTR_{(m+2)} / TTR_{(m+1)})}{(p - 1) + (2 \times (TTR_{(m+2)} / TTR_{(m+1)})}$$

where, p is the number of precursor monomers present in the polymer, p = 8. Finally, our estimate of lipogenesis could be derived by calculating FSR (Wolfe and Chinkes, 2005).

$$FSR_{lipogenesis} = \frac{TTR_{C16:0,\ time1} - TTR_{C16:0,\ time0}}{(p \times MPE_{precursor})\ (1 - MPE_{precursor})^{p-1}\ (time1 - time0)}$$

Desaturation**.** Stearoyl-CoA desaturase 1 (SCD1) is the enzyme responsible for creating a double bond at the Δ^9 position of several fatty-acyl CoA and its preferred substrates are C16:0 and C18:0 (Enoch et al., 1976). Its transcription and activity can regulated by certain fatty acids (Ntambi, 1995), including palmitoleic acid (Burns et al., 2010). In order to measure desaturation rates *in vitro*, we added 100 μM U-^{13}C18:0 to cultures treated with 0 μM fatty acid (control) or 150 μM palmitic, palmitoleic, or *cis*-vaccenic acid on d 6 for 0, 12, and 24 h. Samples were prepared and analyzed as described above for analysis with GC-MS. Ions of mass-to-charge ratio (*m*/*z*) 298 (*m*) and 316 (*m* + 18) were selectively measured to calculate the isotopic enrichments of C18:0. Similarly, isotope abundance of 296 (*m*) and 314 (*m* + 18) was measured for C18:1 *cis*-9. Since tracer enrichment can be measured in this case and the product of the SCD reaction is not a polymer of the tracer, it was not necessary to use MIDA. The FSR was calculated for rate of desaturation using the precursor enrichment, $MPE_{C18:0}$.

$$FSR_{desaturation} = \frac{TTR_{C18:1\ cis\text{-}9,\ time1} - TTR_{C18:1\ cis\text{-}9,\ time0}}{MPE_{C18:0} \times (time1 - time0)}$$

Gene Expression. Total cellular RNA was isolated from cells using the *mir*Vana microRNA Isolation kit (Ambion, Austin, TX) according to manufacturer's instructions and RNA quality as described by Duckett et al. (2009). Quality was assessed using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and Agilent Bioanalyzer 2100. All tcRNA samples used in real-time PCR had a 260:280

absorbance ratio > 1.9 on the Nanodrop and RNA integrity number > 9.0 (1.0 to 10.0 scale) using Agilent RNA 6000 Nano kit. Superscript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA) was used to synthesize first strand cDNA. Real-time PCR was conducted using an Eppendorf MasterCycler ep realplex (Westbury, NY) with the QuantiTect SYBR Green RT-PCR Two Step Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Two genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, were evaluated as housekeeping genes for data normalization (Duckett et al., 2009). To determine the appropriate housekeeping gene to be used to normalize the data, the cycle threshold values (C_T) for GAPDH, β-actin, and all target genes per sample were entered into the BESTKEEPER program (http://www.gene-quantification.info). The program determines the most stable housekeeping gene to be used for normalization by repeated pair-wise correlation and regression analysis (Pfaffl et al., 2002). Both GAPDH and β-actin exhibited a correlation coefficient of 0.99 ($P <$ 0.001) in the analysis and were suitable for data normalization. Primers for bovine mRNAs were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Primer sets were first evaluated according to Duckett et al. (2009) to verify identity. Genes of interest for this study were those involved in fatty acid biosynthesis including acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), fatty acid elongase (ELOVL) -5 and -6, SCD1, sterol regulatory element binding protein-1c (SREBP), and carnitine palmitoyl-transferase 1A (CPT1A).

Statistical Analysis. The data were analyzed using Proc GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC) for treatment comparisons over time. A two-way

analysis of variance (ANOVA) was performed as a completely randomized design with factors fatty acid (control, palmitic, palmitoleic, and *cis*-vaccenic), time (0, 12, 24, and 36 h), and two-way interaction. Least squares means were computed and separated statistically using Fisher's Protected LSD test. Relative gene expression data was analyzed using Pair-wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002) with preplanned treatment comparisons.

RESULTS AND DISCUSSION

Experiment 1. Incubating bovine adipocytes with U-^{13}C16:1 resulted in enrichment ($P < 0.001$) of C16:1 and C18:1 *cis*-11 fatty acids over time. Tracer-to-tracee ratio of C16:1 was greater than zero ($P < 0.01$) 6 h after U-^{13}C16:1 addition to the media and remained elevated ($P < 0.01$) to 36 h (Figure 1). Enrichment of C18:1 *cis*-11 as TTR was also greater than zero ($P < 0.05$) by 6 h and continued to increase ($P < 0.01$) above 6 h levels at 24 and 36 h. The presence of tracer definitively shows that C16:1 is taken in by bovine adipocytes and elongated to C18:1 *cis*-11 in palmitoleic acid-supplemented cells. When adipocytes were exposed to unlabeled palmitoleic acid from d 2 to d 6 and U-^{13}C16:1 on d 6, the $FSR_{elongation}$ did not change ($P > 0.05$) over the 36 h period and was lower ($P < 0.01$) throughout the period compared with control cells. The $FSR_{elongation}$ increased ($P < 0.05$) over the 36 h period in control adipocytes, those cells which were newly exposed to palmitoleic acid (Figure 1).

In addition to label appearing in C16:1 and C18:1 *cis*-11 fatty acids following incubation with U-^{13}C16:1, enrichment was seen in C20:1 *cis*-13 and CLA *cis*-9, *cis*-11 (data not shown). Statistical analysis was not performed because fatty acid peaks for C20:1 *cis*-13 and CLA *cis*-9, *cis*-11 were only detectable in a few samples. However, when detectable, fatty acid peaks contained masses consistent with $m + 16$ enrichment from U-^{13}C16:1. The formation of these fatty acids is possible through established fatty acid pathways. Once palmitoleic acid is elongated to C18:1 *cis*-11, it can be further elongated to C20:1 *cis*-13 or desaturated by SCD1 to CLA *cis*-9, *cis*-11.

Experiment 2. We successfully incorporated our fatty acid supplements into bovine adipocyte cultures based on detection of increased levels ($P < 0.01$) of treatment fatty acids in cells after all media was removed. Comparing fatty acid data between control, palmitic, palmitoleic, and *cis*-vaccenic acid-treated cells, there was a main effect of fatty acid treatment ($P < 0.01$) for total fatty acids (Table 1). Micrograms of total fatty acids were greatest ($P < 0.001$) in palmitoleic and *cis*-vaccenic acid-treated cells at about 2X greater than control cells (Table 1). Palmitic acid-treated cells also had higher ($P < 0.05$) levels of total fatty acids compared with controls, but lower ($P < 0.05$) than palmitoleic and *cis*-vaccenic acid-treated cells, despite being supplemented at the same level. Reduced total fatty acids in palmitic acid-treated cells compared with MUFA-treated cells is most likely due to an apoptitic effect of palmitic acid supplementation. In murine adipocytes, palmitic acid induces apoptosis (Guo et al., 2007). Similarly, we suspected that palmitic acid-supplementation in our bovine adipocyte cultures induced apoptosis, resulting in reduced total lipid accumulation and treatment fatty acid inclusion compared with MUFA-supplemented cells. To test this hypothesis, we treated differentiated adipocytes with palmitic and palmitoleic acid to d 6 and assayed cell viability using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD) according to manufacturer's instructions. Relative cell viability was greater ($P < 0.05$) for palmitoleic acid-treated cells compared with palmitic acid-treated cells (data not shown). Despite a reduction in cell viability, palmitic acid-treated cells had increased ($P < 0.05$) levels of C16:0, C18:0, C18:1 *cis*-9, and C18:2n-6 compared with all other treatment groups.

Palmitoleic acid-treated cells had increased ($P < 0.05$) levels of C16:1 compared with all other treatment groups. In addition, palmitoleic acid cells had increased ($P < 0.05$) levels of C18:1 *cis*-11 and C20:1 *cis*-13 compared with controls, consistent with results from our previous work . *Cis*-vaccenic acid-supplemented cells had slightly elevated ($P < 0.05$) C16:1 compared with controls, but were not different ($P > 0.05$) than palmitic acid-treated cells. Also, *cis*-vaccenic acid-supplemented cells had the greatest ($P < 0.05$) amount of C18:1 *cis*-11 and C20:1 *cis*-13 compared with all other treatment groups.

Palmitic acid-treated cells had the greatest ($P < 0.05$) levels of C18:0, C18:1 *cis*-9, and C18:2n-6 levels compared with all other treatment groups (Table 1). Palmitoleic and *cis*-vaccenic acid-treated cells had the lowest ($P < 0.05$) levels of C18:1 *cis*-9 and C18:2n-6 fatty acids. Despite low levels of C18:1 *cis*-9 in both MUFA-treated groups, the desaturation index of C18:1 *cis*-9/C18:0 was reduced ($P < 0.05$) in palmitoleic acid treated cells only compared will all other treatment groups (Table 1). The desaturation index of C18:1 *cis*-9/C18:0 indicates desaturase activity was only impacted by palmitoleic acid supplementation, not *cis*-vaccenic acid. However, supplementation of either MUFA in adipocyte cultures probably resulted in altered fatty acid metabolism of long chain-PUFA, as C18:2n-6 was decreased ($P < 0.05$) in both groups compared with controls and C20:4n-6 tended ($P = 0.07$) to be lower.

Lipogenesis. Precursor enrichment, $MPE_{acetate}$, did not change ($P > 0.05$) over time or due to treatment in our cultures using MIDA, nor was there a time by treatment interaction (Figure 2), suggesting we had isotopic steady state in our cultures (Martini et

al., 1999). There was an interaction ($P < 0.01$) between fatty acid treatment and length of ^{13}C2 incubation on C16:0 enrichment. The $TTR_{C16:0}$ did not differ ($P > 0.05$) between treatment groups at 0 h and increased ($P < 0.05$) in all groups over time. At 24 and 36 h time points, $TTR_{C16:0}$ was decreased ($P < 0.05$) in palmitoleic and *cis*-vaccenic-treated cells compared with controls. The $FSR_{lipogenesis}$, calculated from $MPE_{precursor}$ and $TTR_{C16:0}$, also shows a reduction in lipogenesis in palmitoleic and *cis*-vaccenic acid-treated cells. The $FSR_{lipogenesis}$ was decreased ($P < 0.01$) in MUFA-treated cells compared with controls between the 24 and 36 h time points and was numerically lower between 0 to 12 h and 12 to 24 h time points (Figure 2). Palmitoleic acid-treated cells tended to have a lower ($P = 0.07$) $FSR_{lipogenesis}$ between 0 and 12 h time points. Reduced lipogenesis in palmitoleic acid-treated cultures is consistent with our previous results when we supplemented 150 μM palmitoleic acid, which resulted in approximately 45% reduction in lipogenesis. In this case, both MUFA induced a repressive effect on lipogenesis of approximately the same magnitude. Therefore, this effect of palmitoleic acid supplementation on lipogenesis cannot be directly attributed to palmitoleic acid as its elongated form, *cis*-vaccenic acid, also displayed anti-lipogenic effects.

Desaturation. Because the desaturation index of C18:1 *cis*-9/C18:0 is not always accurate (Archibeque et al., 2005), we supplemented a labeled substrate of the SCD1 reaction (U-^{13}C18:0) to our bovine adipocyte cultures to serve as a measurement of desaturase activity. There was a treatment by length of incubation interaction for precursor enrichment, $TTR_{C18:0}$. It was not different ($P > 0.05$) than zero at 0 h, but $TTR_{C18:0}$ increased ($P < 0.05$) over time for each sample. However, there was a

differential incorporation of labeled U-^{13}C18:0 into the different treatment groups. Cells treated with palmitoleic and *cis*-vaccenic acids had increased ($P < 0.01$) $TTR_{C18:0}$ compared with control and palmitic acid-treated cells (Figure 3). There was also a treatment by incubation time interaction ($P < 0.01$) for $TTR_{C18:1\ cis\text{-}9}$. At 0 h, there was no enrichment ($P > 0.05$) of C18:1 *cis*-9 in any treatment group; by 12 h, $TTR_{C18:1\ cis\text{-}9}$ was still not different than zero in palmitoleic acid-treated cells ($P < 0.01$). However, control, palmitic, and *cis*-vaccenic acid-treated cells did not differ ($P > 0.05$) from each other and were enriched ($P < 0.05$) with C18:1 *cis*-9 at 12 h. By 24 h, all treatment groups differed ($P < 0.05$) from each other and had enrichment ($P < 0.05$) of C18:1 *cis*-9. Palmitoleic acid-treated cells had reduced ($P < 0.01$) $TTR_{C18:1\ cis\text{-}9}$ compared to controls, palmitic, and *cis*-vaccenic acid treated cells at 12 and 24 h. Palmitic and *cis*-vaccenic acid-treated cells had lower ($P < 0.05$) $TTR_{C18:1\ cis\text{-}9}$ at 24 h than controls, but higher ($P < 0.05$) than palmitoleic acid-treated cells. Despite increased $TTR_{C18:0}$ in palmitoleic and *cis*-vaccenic acid-treated cells, they still showed less formation of the desaturase product, C18:1 *cis*-9.

The isotopic measure of SCD1 activity, $FSR_{desaturation}$, showed a more dramatic effect of fatty acid supplementation on desaturase activity than the C18:1 *cis*-9/C18:0 desaturation index results. The $FSR_{desaturation}$ over 0 to 12 h was decreased ($P < 0.01$) in palmitoleic acid-treated cells compared with all other treatment groups, which did not differ ($P > 0.05$) from each other (Figure 3). From 12 to 24 h, however, all treatment groups had a different $FSR_{desaturation}$. Palmitic acid-treated cells had the highest ($P < 0.01$) rate of desaturation and palmitoleic acid-treated cells had the lowest ($P < 0.01$) $FSR_{desaturation}$ from 12 to 24 h (Figure 3). The reduction in desaturase activity due to

palmitoleic acid was approximately 100% from 0 to 12 h and approximately 60% from 12 to 24 h. By contrast, palmitoleic acid reduced the desaturation index by less than 25% compared with controls (Table 1). The $FSR_{desaturation}$ also showed a reduction in SCD1 activity by approximately 15% from 12 to 24 h due to *cis*-vaccenic acid (Figure 3), whereas the desaturation index for *cis*-vaccenic acid-treated cells was not different from controls. Therefore, the $FSR_{desaturation}$ is a more sensitive measurement of SCD1 activity than the C18:1 *cis*-9/C18:0 desaturation index when fatty acids are supplemented to adipocyte cultures.

Gene Expression. The genes that primarily involved in *de novo* lipogenesis are ACC and FASN. In this study, palmitic acid-treated cells did not differ ($P > 0.05$) from control cultures for ACC or FASN mRNA expression. Acetyl-CoA carboxylase was up-regulated ($P < 0.05$) in response to both palmitoleic and *cis*-vaccenic acid supplementation compared with controls (Figure 4). In contrast, FASN was down-regulated ($P < 0.05$) by both MUFA. These 2 results are seemingly contradictory. However, ACC activity can be regulated also be regulated allosterically and through phosphorylation. Regulated by the transcription factor SREBP, FASN gene expression was reduced by approximately 40%, similar to $FSR_{lipogenesis}$ results.

Transcription of SCD1 mRNA is controlled primarily by SREBP, which binds to sterol response element in the promoter region of the SCD1 gene (Eberle et al., 2004; Lay et al., 2002). In our study, SREBP mRNA was down-regulated ($P < 0.05$) in only palmitic acid-treated cells compared with controls. The promoter region of the bovine SCD1 gene contains a fat specific element, PUFA response element, and SREBP-

response region (Keating et al., 2006). In addition to PUFA, expression of SCD1 is also regulated by its products and not by the availability of its substrates (Keating et al., 2006). For instance, the addition of C18:1 *cis*-9 and CLA *cis*-9, *trans*-11 reduced SCD promoter activity, but C18:1 *trans*-11 did not (Keating et al., 2006). Our study confirmed that palmitoleic acid, and not *cis*-vaccenic acid, acts to down-regulates ($P < 0.05$) SCD1 gene expression in bovine cells (Figure 4). Palmitic acid-treated cells were not different ($P > 0.05$) from controls in SCD1 mRNA expression. Both the desaturase index and $FSR_{desaturase}$ confirm that palmitoleic acid impacts actual activity of the enzyme, not just gene expression; whereas, *cis*-vaccenic acid induces a minimal response in rate of desaturation with no effect on the desaturation index or gene expression and palmitic acid increases the rate of desaturation with no effect on the desaturation index.

Several elongase enzymes are present in mammalian cells and are responsible for adding acetyl-CoA molecules to the carboxylic acid end of a fatty acid hydrocarbon skeleton. Each elongase isoform has preferred fatty-acyl substrates of particular C length (Guillou et al., 2010). In humans, most ELOVL proteins identified are responsible for elongating PUFA greater than or equal to 18C; however, ELOVL6 is attributed to the conversion of 12 to 16C SFA and MUFA, including C16:1 to C18:1 *cis*-11 (Matsuzaka et al., 2002; Leonard et al., 2004). It has been proposed that C16:1 *cis*-9 can be elongated into C18:1 *cis*-11 and potentially elongated further into a 20C fatty acid, C20:1 *cis*-13 (Matsuzaka and Shimano, 2009). In addition, ELOVL5 is reported to have some activity for this reaction (Wang et al., 2006), especially in absence of ELOVL6 (Matsuzaka et al., 2007). As FASN is not capable of synthesizing fatty acids greater than 16C in length,

acetate molecules were most likely added to C16:1 *cis*-9 by either ELOVL5 or -6 in our study. Primarily regulated by lipogenic transcription factors (Leonard et al., 2004; Wang et al., 2006), these 2 ELOVL isoforms are the only bovine elongase enzymes published on NCBI to date (Zimin et al., 2009). Transcription factors that regulate ELOVL6 include SREBP-1c and peroxisome proliferator-activated receptor γ (PPARγ); ELOVL5 expression is up-regulated PPARγ and not by SREBP-1c (Wang et al., 2006). In our study, expression of ELOVL6 was down-regulated ($P < 0.05$) with palmitic, palmitoleic, and *cis*-vaccenic acid supplementation; however, ELOVL5 expression was up-regulated ($P < 0.05$) with MUFA treatments (Figure 4). Palmitic acid-treated cells had numerically lower ELOVL5 mRNA expression compared with controls.

In our adipocyte cultures, CPT1A mRNA was up-regulated ($P < 0.05$) by all fatty acid treatments and tended ($P = 0.10$) to be highest in palmitoleic acid-treated cells. Carnitine palmitoyl-transferase 1A is responsible for transporting long chain fatty acids through the outer mitochondrial membrane for the purpose of β-oxidation in the mitochondrial matrix. In ruminants, CPT1A is expressed in numerous tissues including adipose tissue in contrast to a more restricted tissue abundance in monogastrics (Price et al., 2003). Inhibition of CPT1A is primarily attributed to malonyl-CoA, the product of the ACC reaction, in monogastric species (McGarry and Brown, 1997). Price et al. (2003) found N-terminal sequence differences in ovine CPT1 that alter enzyme kinetics for certain substrates, which differs from that of the rat. Palmitoleic acid supplementation stimulates CPT1A transcription which may lead to increase β-oxidation and ATP production.

In this study, we confirmed C18:1 *cis*-11 and C20:1 *cis*-13 as products of palmitoleic acid elongation *in vitro* and that CLA *cis*-9, *cis*-11 can also be produced through elongation and desaturation of palmitoleic acid. Palmitoleic acid and *cis*-vaccenic supplementation reduces lipogenesis and FASN gene expression. However, palmitoleic acid only reduces SCD1 gene expression and desaturation activity. Therefore, palmitoleic acid is actively elongated *in vitro* and its elongation product, *cis*-vaccenic, can also reduce lipogenesis, but only palmitoleic acid can reduce desaturation rates.

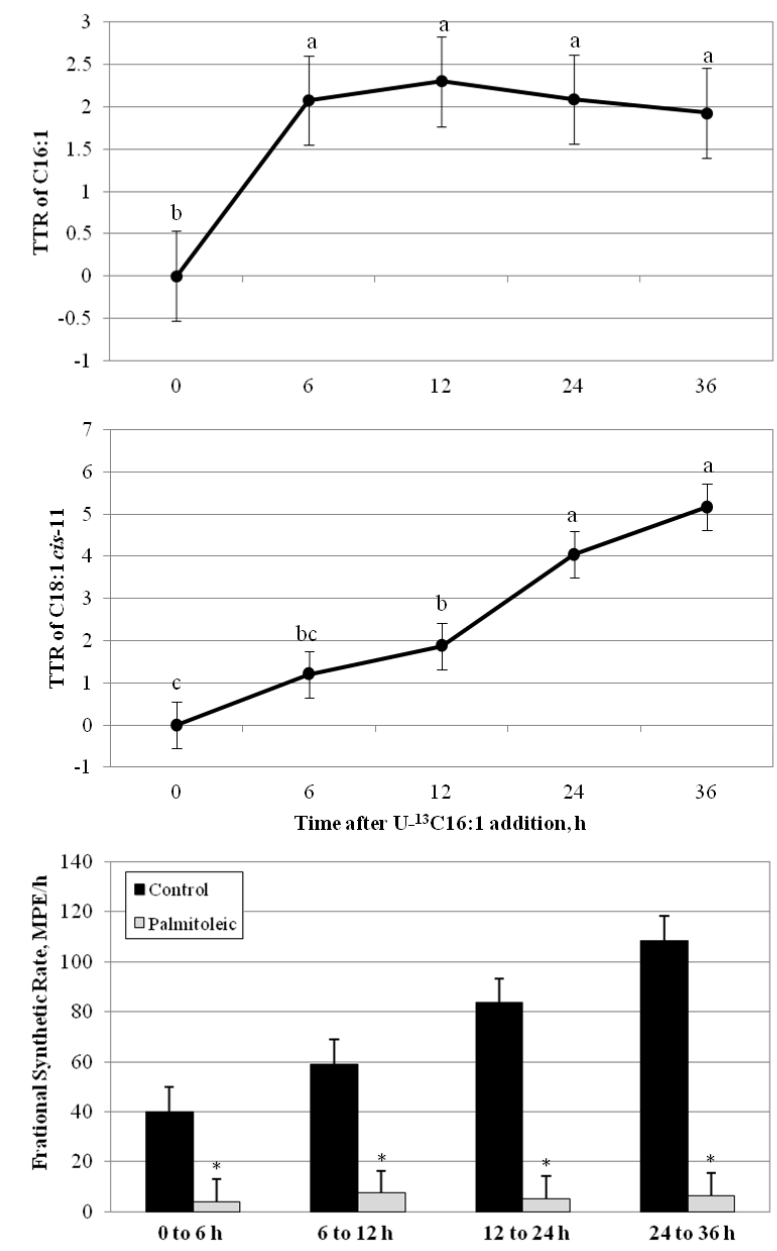

Figure 1. Elongation of C16:1 to C18:1 *cis*-11 in bovine adipocyte cultures. Tracer-to-tracee ratio (TTR) of C16:1 and C18:1 *cis*-11 in displayed over time in control cells after 150 μM U-^{13}C16:1 addition. Fractional synthetic rate of ^{13}C18:1 *cis*-11 from ^{13}C16:1 in cells treated with 0 μM (control) or 150 μM palmitoleic acid until d 6 displayed as molar percent excess (MPE) per h and was calculated over a 36 h period following stable isotope administration. $^{a\text{-}c}$Time points without a common letter differed ($P < 0.05$). *Within a time point, palmitoleic acid-treated cells differed ($P < 0.05$) from controls.

Table 1. Fatty acid composition of bovine adipocyte cultures treated with 0 μM fatty acids (control), 150 μM palmitic, palmitoleic, or *cis*-vaccenic acid.

		150 μM				
Fatty acid, μg/well	Control	Palmitic	Palmitoleic	*Cis*-Vaccenic	SEM	*p*-value
C16:0	8.99^{cd}	17.57^{a}	10.45^{bc}	8.53^{d}	0.49	0.0006
C16:1 *cis*-9	0.55^{c}	1.55^{bc}	31.88^{a}	2.8^{b}	2.33	< 0.0001
C18:0	10.19^{b}	12.07^{a}	9.74^{b}	7.24^{c}	0.32	0.0022
C18:1 *cis*-9	11.4^{b}	13.2^{a}	8.15^{c}	8.18^{c}	0.35	0.0012
C18:1 *cis*-11	3.27^{c}	3.94^{c}	41.56^{b}	70.39^{a}	2.71	0.0054
C18:2n-6	1.76^{b}	1.93^{a}	1.48^{c}	1.54^{c}	0.04	< 0.0001
C20:1 *cis*-13	ND^{c}	0.15^{c}	3.29^{b}	3.95^{a}	0.11	< 0.0001
C20:4n-6	6.42	6.65	6.11	5.84	0.15	0.0676
Total fatty acids	67.04^{c}	89.98^{b}	145.37^{a}	139.95^{a}	4.65	0.0007
C16:1/C16:0	0.06^{c}	0.09^{c}	3.05^{a}	0.33^{b}	0.06	< 0.0001
C18:1 *cis*-9/C18:0	1.12^{a}	1.09^{a}	0.84^{b}	1.13^{a}	0.03	0.0091

$^{a-d}$ Within a row, means without a common superscript differ ($P < 0.05$)

ND, fatty acid not detected

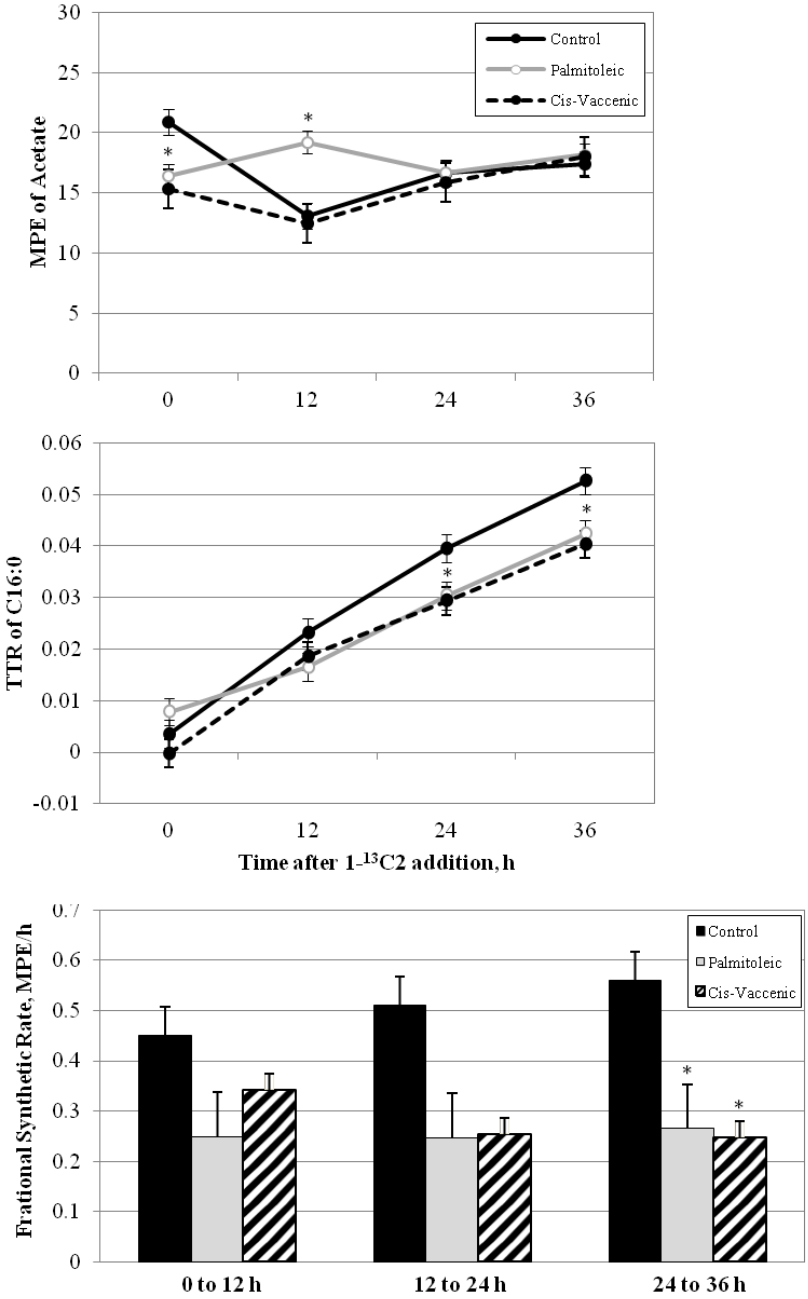

Figure 2. Lipogenesis in bovine adipocyte cultures treated with 0 μM fatty acid (control), 150 μM palmitoleic or *cis*-vaccenic acid. Estimation of molar percent excess (MPE) of acetate after 1-^{13}C2 addition to the media. Enrichment of C16:0 displayed as tracer-to-tracee ratio of C16:0 over time. Fractional synthetic rate of ^{13}C16:0 from ^{13}C2 displayed as molar percent excess (MPE) per h calculated over a 36 h period following stable isotope administration. *Within a time point, fatty acid-treated cells differed ($P <$ 0.05) from controls.

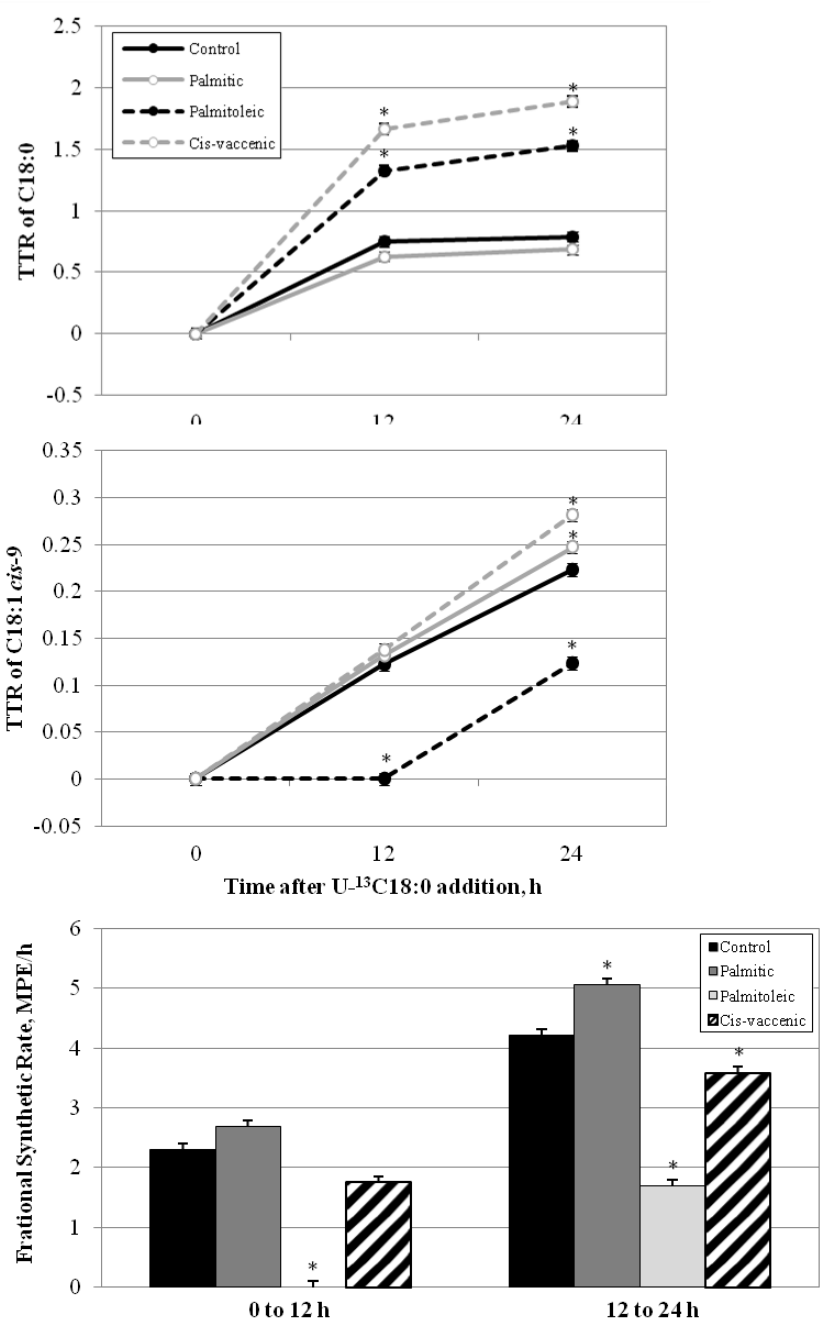

Figure 3. Desaturation of C18:0 to C18:1 *cis*-9 in bovine adipocyte cultures treated with 0 μM fatty acids (control), 150 μM palmitic, palmitoleic, or *cis*-vaccenic acid. Tracer-to-tracee ratio (TTR) of C18:0 and C18:1 *cis*-9 displayed over time following 100 μM U-^{13}C18:0 addition to the media. Fractional synthetic rate of ^{13}C18:1 *cis*-9 from ^{13}C18:0 displayed as molar percent excess (MPE) per h calculated over a 24 h period following stable isotope administration. *Within a time point, fatty acid-treated cells differed (P < 0.05) from controls.

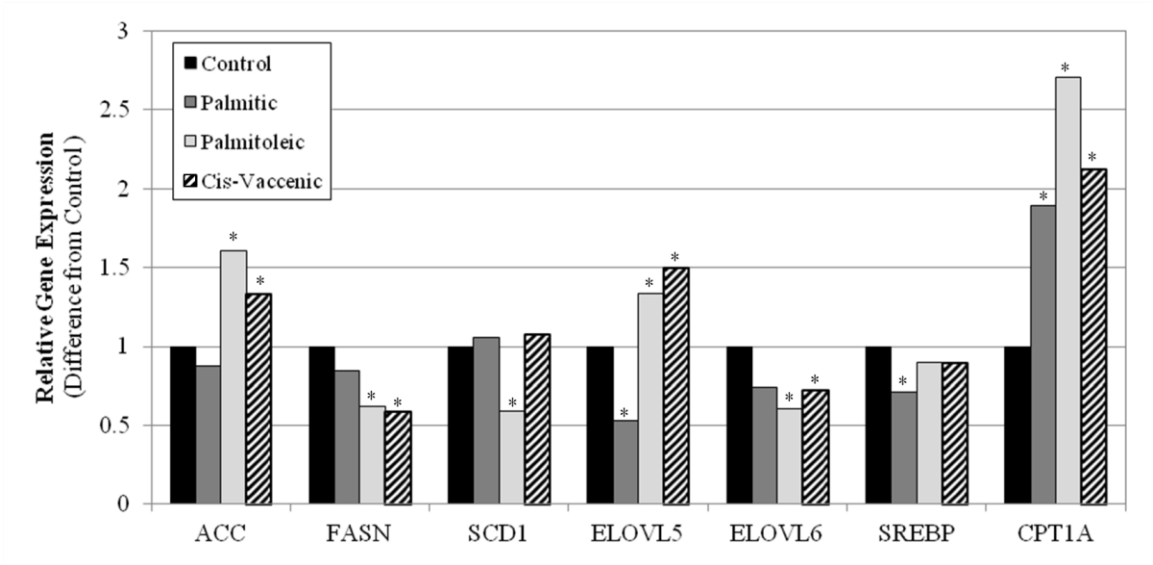

Figure 4. Relative expression of genes associated with lipogenesis in bovine adipcytes supplemented with 0 μM fatty acids (control) or 150 μM palmitic, palmitoleic or *cis*-vaccenic acid. Acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), fatty acid elongase (ELOVL)-5 and -6, sterol response element binding protein-1c (SREBP), and carnitine-palmitoyl transferase 1A (CPT1A) were up-regulated in adipcytes exposed to MUFA supplements. All C_T values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Gene expression in fatty acid-treated cells differed ($P < 0.05$) from controls.

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