

**STEAROYL-COA DESATURASE GENE TRANSCRIPTION, mRNA,
AND ACTIVITY IN RESPONSE TO *TRANS*-VACCENIC ACID AND
CONJUGATED LINOLEIC ACID ISOMERS**

by

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State
University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

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August 15, 2000

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Key words: lipogenesis, mammary cells, stearoyl-CoA desaturase, *trans*-vaccenic acid, conjugated linoleic acid, transcription

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(ABSTRACT)

Studies were conducted to investigate: 1) desaturation of dietary *trans*-vaccenic acid (TVA, *trans*11-18:1) to the *cis*9,*trans*11-18:2 isomer of conjugated linoleic acid (9/11CLA), 2) effects of two conjugated linoleic acid isomers [9/11CLA or *trans*10,*cis*12-18:2 (10/12CLA)] and TVA on enzyme activities and mRNA abundance for lipogenic enzymes, and 3) regulation of stearoyl-CoA desaturase (SCD) gene transcription. In the first study, lactating mice were fed 3% linoleic acid (LA), or 2% LA plus 1% stearic acid (SA), 1% TVA, or 1% CLA mixture. Dietary TVA enriched the 9/11CLA content of carcass, liver, and mammary tissue of lactating mice. A similar enhancement of 9/11CLA also was observed in liver, but not carcass, of suckling pups nursing TVA-fed dams. The CLA mixture decreased mammary acetyl-CoA carboxylase (ACC) activity compared with other treatments. However, total fatty acid content of mammary tissue was reduced only when compared with TVA. In the second experiment, lactating mice were fed 3% canola oil (OA), or 2% OA plus 1% SA, 1% TVA, 1% 9/11CLA, or 1% 10/12CLA. Dietary TVA, 9/11CLA, and 10/12CLA decreased mRNA abundance for ACC and fatty acid synthase (FAS) in mammary tissue, suggesting each had the potential to reduce *de novo* fatty acid synthesis. However, only the CLA isomers decreased ACC activity in mammary tissue and concentration of medium-chain fatty acids (MCFA = 12:0+14:0+16:0) in milk fat. The 10/12CLA isomer caused greater reductions in MCFA and milk fat percentage than the 9/11CLA, indicating that 10/12CLA is the primary CLA isomer affecting lipid metabolism in the mammary gland. Dietary TVA, 9/11CLA, or 10/12CLA decreased SCD enzyme activity and mRNA abundance in mammary tissue. In study 3, mouse (COMMA-D/MME) and bovine (Mac-T) mammary epithelial cells were transfected with the putative promoter (600 bp) of SCD gene. The 9/11CLA reduced SCD gene transcription in mouse cells, but not bovine cells. Transcription, however, was reduced in both cell lines by 10/12CLA, linoleic acid, and linolenic acid. Thus, reduced SCD transcription in response to the CLA isomers in mouse mammary cells *in vitro* may provide an explanation for reduced SCD enzyme activity and mRNA abundance in mammary tissue when lactating mice were fed either of the CLA isomers. In contrast, stearic acid, oleic acid, and TVA did not affect SCD transcription. Although TVA did not reduce SCD transcription in mouse mammary cells *in vitro*, it

did reduce SCD enzyme activity and mRNA abundance in mammary tissue when fed to lactating mice. The results suggested TVA may influence SCD mRNA processing or stability in the nucleus after transcription. Despite the reduction in SCD mRNA and enzyme activity, however, substantial quantities of TVA were desaturated to the 9/11CLA isomer when TVA was fed to lactating mice in the first two studies. Thus, dietary TVA provides an alternate supply of the anticarcinogenic 9/11CLA isomer in tissues.

ACKNOWLEDGEMENTS

It is the most exciting moment for me to express my sincere gratitude to all people who have made my program successful. I sincerely acknowledge the John Lee Pratt Fellowship in Animal Nutrition for providing financial support for my program of study.

Special thanks go to Dr. Joseph H. Herbein for his guidance and support as my major advisor. His patience, kindness, and encouragement for me are very much appreciated. I would like to thank Dr. Eric A. Wong for the valuable training and guidance and for giving me the privilege of using the facilities in his laboratory during the research. I would like to extend my sincere gratitude to Dr. Ronald E. Pearson for his insightful criticisms, guidance in statistical analysis, and for his valuable also enjoyable time as a committee member. My sincere thanks go to Dr. Thomas W. Keenan for his kindness in allowing me to use his facilities for the first enzyme assays, for providing insights to many questions from me, and for all the enjoyable conversations. My sincere gratitude also is extended to Dr. Charles L. Rutherford for providing his expertise and for his valuable time as a committee member. I would like to thank Dr. W. E. Vinson for leading such a good and big family in which I am the only Chinese student.

Many thanks go to Ms. Wendy Wark for her daily support and her encouragement. I would like to thank Ms. Pat Boyle for enhancing my cell culture techniques and for providing assistance with many requests from me. I also would like to give my sincere thanks to Mr. Lee Johnson for sharing with me his amazing knowledge and skills relating to radioactive assays. Thanks to Ms. Kerry Waite and Ms. Kingsley Weaver for valuable information and help in molecular biology techniques. Thanks to Mr. Gene Ball for his assistance in the protein assay. I sincerely thank Dr. Kenneth E. Webb for permitting me to use his scintillation counter for the enzyme assays.

Thanks to Dr. Yuanxiang Pan and Ms. Hong Chen for their friendship, support, and encouragement. Both of them are always ready to answer my endless questions and to help me with any new procedures. My sincere thanks go to Dr. Geetha C. Jayan for teaching me cell culture techniques and for starting my research project. Thanks to Dr. Aloka B. P. A. Bandara for his friendship and encouragement. I would like to give my sincere thanks to Mr. Juan J. Loo for his help, friendship, and encouragement. He is always ready for a lively and rewarding discussion.

Dr. James Ntambi at the University of Wisconsin, Dr. Mark Magnuson at Vanderbilt University, and Drs. Mike Barber and Maureen Travers at Hannah Research Institute, Scotland, are greatly appreciated for donating the cDNA probes for the Northern-blot analysis. I would like to extend my sincere thanks to Dr. James Ntambi also for his generosity in providing the pCAT-SCD promoter. Meanwhile, I am grateful to Dr. R. Michael Akers for kindly sharing the Mac-T cells.

Last but not least, I want to thank my grandma, mom and dad for their endless love and support through so many years. Thanks to my brothers and sister who always give their support. Thanks to my mom- and dad-in-law, my brothers- and sisters-in-law for their encouragement and support. My sincere thanks go to my lively wife, Xiumei Lin, for her love, patience, support, encouragement, and sacrifice. I want to thank my loving daughter, Nancy (Shuonan), for being with me during my study. Her simple presence helps cheer me up always.

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CHAPTER 1

INTRODUCTION

Dietary fat generally has negative health connotations for humans. High intake of saturated fatty acids (SFA) has been implicated in a number of health problems, such as coronary heart disease (CHD). A high intake of *trans* fats also has been associated with an increased risk of heart disease (Willett et al., 1993). On the other hand, unsaturated fatty acids (UFA) are associated with lower plasma low-density lipoprotein-cholesterol, the 'bad' cholesterol that increases the risk of CHD (Grundy et al., 1988).

Milk was described as 'the most nearly perfect food' (Ensminger, 1993). It provides many nutrients that are easily digested and absorbed. Of these, milk fat is an important source of essential fatty acids. Milk fat also contains fat-soluble vitamins. However, bovine milk fat contains more than 70% SFA, relatively low concentrations of UFA, and significant amounts of *trans* fatty acids. Therefore, the dairy industry should consider practical methods to decrease SFA and increase UFA in bovine milk fat.

Because the major products of de novo fatty acid synthesis in the mammary tissue are SFA, one method to lower SFA is to decrease mammary fatty acid biosynthesis. The primary reason for the low UFA content of bovine milk fat is extensive bacterial hydrogenation of dietary UFA to stearic acid (18:0) in the rumen. To increase UFA in milk fat, either UFA saturation in the rumen must be reduced or stearic acid desaturation in the mammary tissue must be enhanced.

Polyunsaturated fatty acids inhibit lipogenesis in tissues such as liver and adipose tissue. Of more interest, and more relevant, to ruminant nutritionists is the evidence that unsaturated *trans* fatty acids reduced milk fat content and lowered SFA concentration. Wonsil et al. (1994) reported that both dietary and ruminally derived *trans*-18:1 reduced milk fat percentage. Jayan (1998) observed that *trans*-vaccenic acid (TVA, *trans*11-18:1) and conjugated linoleic acid (CLA) reduced saturated fatty acid synthesis in mammary cell cultures by decreasing enzyme activity and mRNA levels for acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Jayan and Herbein, 1999). Loor and Herbein (1998) reported that a CLA mixture [primarily *cis*9,*trans*11-18:2 (9/11CLA) and *trans*10,*cis*12-18:2 (10/12CLA)] reduced concentration and yield of SFA in milk by decreasing de novo synthesis of SFA in the mammary gland of dairy cows. However, it remains unknown whether one isomer of CLA or both reduced de novo synthesis.

A side effect of reducing de novo SFA synthesis through an enhanced supply of unsaturated *trans* fatty acids is the inhibition of stearoyl-CoA desaturase (SCD). The mixture of CLA reduced concentration and yield of SFA but also inhibited the desaturation of 18:0 (Loor and Herbein, 1998). Similarly,

Chouinard et al. (1999a) reported a mixture of CLA reduced the percentage and yield of fat in milk by 28% and 25%, respectively. The CLA mixture also inhibited desaturation of 18:0 in their study. In contrast, Jayan and Herbein (1999) observed that TVA and CLA enhanced enzyme activity and mRNA levels of SCD in bovine mammary cell cultures. However, it is still unclear which isomer of CLA affects de novo synthesis and the desaturation process. At the same time, the mechanism by which *cis* and *trans* isomers of 18:1 and 18:2 regulate SCD gene transcription is still not known.

The CLA and TVA found in milk fat are intermediates produced in biohydrogenation of dietary UFA in the rumen. Interestingly, an increase in the content of 9/11CLA in bovine milk by special feeding conditions (pasture feeding, for example) is always associated with an increase in TVA, where the correlation (*r*) between 9/11CLA and TVA is 0.98 (Molkentin, 1999). The potential value of bovine milk fat is enhanced by its TVA content, because in vitro studies have demonstrated that rat liver microsomes desaturated TVA to 9/11CLA (Pollard et al., 1980). Synthesis of 9/11CLA also was observed when TVA was supplemented in mouse and bovine mammary epithelial cell cultures (Jayan, 1998). An 11% conversion of dietary TVA was reported in growing mice and it was postulated that conversion occurred in adipose tissue (Santora et al., 2000).

Objectives of the present study were:

1. To determine the extent to which 9/11CLA could be enriched in tissues of lactating mice and their suckling pups by dietary TVA,
2. To evaluate effects of specific CLA isomers (9/11CLA and 10/12CLA) on enzyme activity and mRNA abundance for ACC, FAS, and SCD, and
3. To investigate effects of stearic acid, linolenic acid, and *cis* or *trans* isomers of 18:1 or 18:2 on transcription of the SCD gene.

CHAPTER 2

LITERATURE REVIEW

Fatty acids

Fatty acid classification and nomenclature

Fatty acids are a class of aliphatic acids, consisting of a long hydrocarbon chain ending in a carboxyl group. They can be divided into saturated fatty acids (SFA), with no double bonds and unsaturated fatty acids (UFA), with one (monounsaturated fatty acids: MUFA) or more (polyunsaturated fatty acids: PUFA) double bonds. When double-bonded carbon atoms in a PUFA alternate with a single bond (-C=C-C=C-), the acid is referred to as conjugated. The configuration of a double bond can be either *cis* or *trans*, depending on the relative positions of the alkyl groups: *cis* (on the same side), *trans* (on different sides).

Fatty acids have important physiologic roles. They are building blocks of phospholipids and glycolipids, which in turn are important components of biological membranes. Many proteins are targeted to membrane locations by covalently attached fatty acids. Fatty acids stored as triglycerides can serve as a source of energy between meals. Some fatty acid derivatives serve as hormones and intracellular messengers.

The traditional names of fatty acids are based on the names of the botanical or zoological species from which they were isolated. Such names may cause some confusion because they provide no clue to the structure of the acid. Systematic nomenclature identifies a fatty acid and describes its structure on the basis of the number of carbon atoms and the number and position of unsaturated bonds relative to the carboxyl group(s). The substituted groups and their positions are identified and the optical activity and geometric configuration at double bonds also are designated. However, modifications to this naming system have evolved and resulted in different naming systems. Trivial names indicating the source of fatty acids are widely used. One example is stearic acid from the Greek *stear*, meaning tallow. Semi-systematic nomenclature retains some of the structural features of systematic nomenclature while using trivial names. The structural system identifies fatty acids by carbon number and number of unsaturated bonds. For example, *cis9,cis12,cis15*-linolenic acid is designated 18:3 Δ^9 , which also indicates the number of the carbon where the first double bond begins (Lobb and Chow, 2000).

In this and following chapters, stearic acid will be designated 18:0, containing 18 carbons without a double bond. Oleic acid will be *cis9*-18:1, indicating that it has 18 carbons and one *cis* double bond between the ninth and tenth carbons. *Trans*-vaccenic acid contains a *trans* double bond at the eleventh of 18 carbons, and is designated *trans11*-18:1. Linoleic acid will be *cis9,cis12*-18:2, containing 18 carbons and two double bonds at 9th and 12th carbons, respectively. The *cis9,trans11*-conjugated linoleic acid will be *cis9,trans11*-18:2 (or 9/11CLA),

containing 18 carbons and two conjugated double bonds at 9th and 11th carbons, respectively. The *trans*10,*cis*12-conjugated linoleic acid (*trans*10,*cis*12-18:2) (or 10/12CLA) contains 18 carbons and two conjugated double bonds at 10th and 12th carbons, respectively. Linolenic acid will be *cis*9,*cis*12,*cis*15-18:3, containing 18 carbons and three double bonds at 9th, 12th, and 15th carbons, respectively. The position of double bonds is relative to the terminal carboxyl group.

Milk fatty acids: Sources and composition

De novo synthesis in the bovine mammary gland and extraction from blood lipids are the two major sources for milk fatty acids. De novo synthesis of short- and medium- chain fatty acids from acetate and β -hydroxybutyrate (arising from microbial digestion of carbohydrate in the rumen) accounts for about 50% of total milk fatty acids. The fatty acids in bovine blood are a combination of dietary origin, mobilization of adipose tissue triglycerides, or microbial fatty acids synthesized in the rumen (Wu et al., 1991; Chilliard, 1993).

Total lipid content of milk varies considerably among species, ranging from 0% in rhinoceros milk, to 4% in humans and ruminants, to as high as 50% in whales (Neville and Picciano, 1997). Within species, total lipid content depends upon such factors as feeds, stage of lactation, and the amount of body lipids. Triglycerides make up 98% of the lipid in milk. The fatty acid composition of milk lipid also varies among species and is affected by a number of factors. Ruminant milk fat contains appreciable amounts of short-chain fatty acids (4:0 and 6:0), which are absent from most other fats (Van Soest, 1994). Ruminant milk fat also contains significant amounts of medium-chain (8:0 to 16:0) SFA. Both short- and medium-chain fatty acids result from the lipogenic activity of the mammary gland. Ruminant mammary gland is unique in its ability to esterify butyryl-CoA and hexanoyl-CoA into the *sn*-3 position of glycerol, leading to the formation of milk triglycerides enriched with these short-chain fatty acids (Dils, 1986). The predominant product of de novo synthesis is palmitate (16:0), which is released from fatty acid synthase by the long-chain acylthioesterase (thioesterase I). The formation of medium-chain fatty acids results from an altered specificity of chain termination by the medium-chain acylthioesterase (thioesterase II), which terminates elongation of fatty acids before they grow to 16 carbons. The majority of long-chain fatty acids in milk lipid are obtained through uptake of blood lipids by the mammary gland, as evidenced by the fact that feeding protected unsaturated lipid will both increase and alter milk lipid. Typically, bovine milk fat consists of approximately 5% PUFA, 70% SFA, and 25% MUFA (Grummer, 1991). Bovine milk also is a significant source of *trans* fatty acids, in contrast to the fact that most plant UFA are present in the *cis* configuration. The relatively higher SFA, lower PUFA, and the presence of *trans* fatty acids in bovine milk fat are a result of microbial hydrogenation of dietary unsaturated fats in the rumen. Low PUFA in milk also can be attributed to the fact that PUFA are not evenly distributed in the various plasma lipoprotein fractions, but tend to be concentrated in the phospholipids and cholesterol esters of the high-density lipoproteins (HDL) (Dryden et al., 1971). Uptake of fatty acids into the mammary gland from HDL is poor (Brumby et al., 1972) and this also partly explains the low levels of PUFA in milk.

Biohydrogenation of fatty acids in the rumen

The fatty acid composition of dietary fat is significantly different from that of bovine milk fat, which is a result of extensive modification of dietary fatty acids in the rumen. Lipids in the diet are rapidly hydrolyzed by microbes in the rumen. The free fatty acids released are then hydrogenated. Lipolysis is a prerequisite to biohydrogenation, because it is necessary that the UFA have a free carboxyl group (Demeyer et al., 1967).

Biohydrogenation is the process that results in the addition of hydrogen to UFA liberated by lipolysis. The end product of hydrogenation of 18-carbon UFA is 18:0. However, many *cis* and *trans* isomers of monoenoic and dienoic fatty acids are formed as intermediates during the biohydrogenation process. Among them, *trans*-vaccenic acid is quantitatively the most important. The major pathways of biohydrogenation of 18-carbon UFA are shown in Figure 2.1. The extent of hydrogenation of 18-carbon PUFA is high. Doreau and Ferlay (1994) reported that hydrogenation is in most cases between 60 and 95% for linoleic acid, and between 80 and 100% for linolenic acid.

Hydrogenation of polyunsaturated 20- and 22-carbon fatty acids is still uncertain. No hydrogenation was observed in vitro (Ashes et al., 1992). On the contrary, extensive hydrogenation was observed in vivo (Doreau and Chilliard, 1997). The possible metabolic pathways have not been studied.

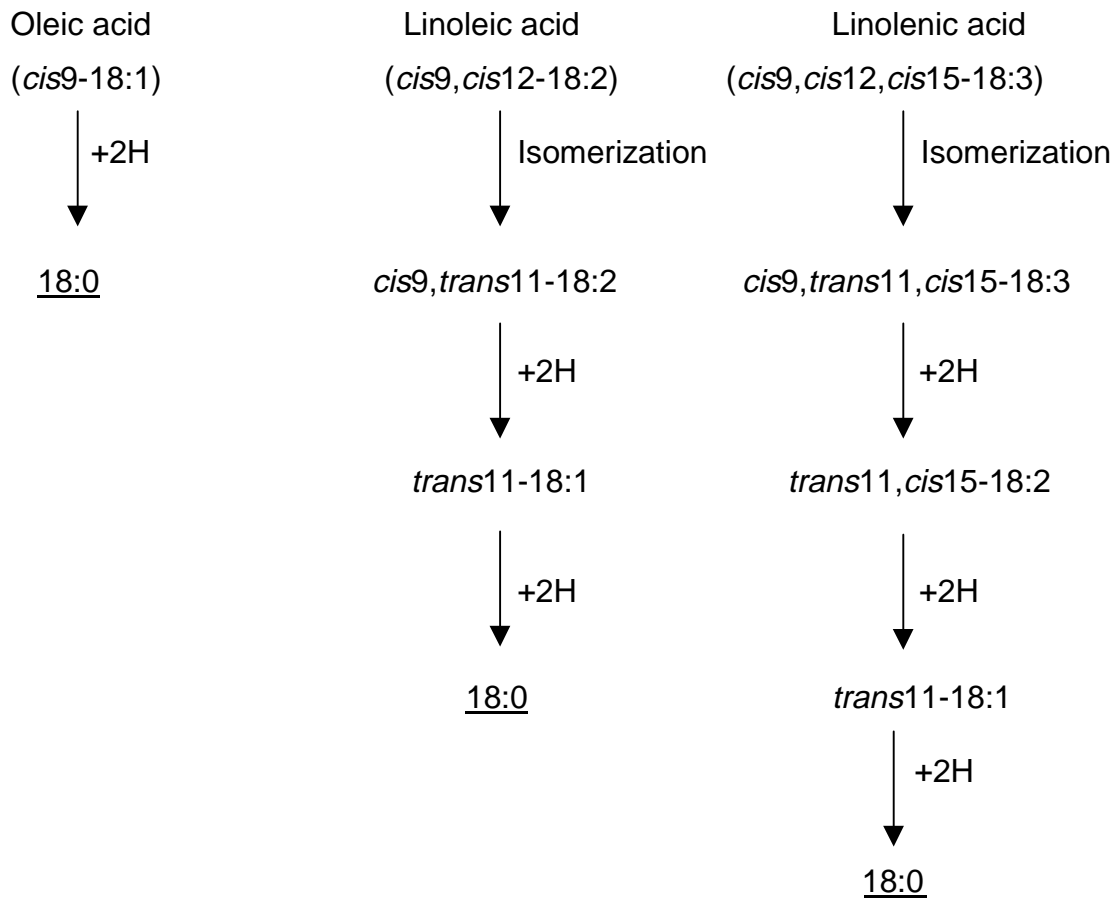


Figure 2.1. Major pathways for UFA biohydrogenation in the rumen.
(Adapted from Kellens et al., 1986)

Desaturation of SFA in the mammary gland

From the previous section it is clear that bovine milk fat contains short- and medium-chain fatty acids synthesized de novo and long-chain fatty acids of dietary and microbial origins. Due to biohydrogenation of dietary UFA by rumen microbes, bovine milk fat is characterized by fatty acids more saturated than the diet (Jenkins, 1994).

The desaturation process introduces double bonds into a SFA. During passage into the mammary gland, triglyceride is largely or completely hydrolyzed by a lipoprotein lipase. There is little further modification of preformed fatty acids within the mammary gland except for extensive desaturation of medium- and long-chain SFA, particularly 18:0 (Mansbridge and Blake, 1997). For example, Storry (1981) reported that increased intake of 14:0, 16:0, and 18:0 led to increases in the corresponding *cis*-MUFA in milk. Therefore, activity of microsomal desaturase enzyme may reverse the extensive ruminal hydrogenation of dietary UFA (Bickerstaffe and Annison, 1969), facilitate milk triglyceride synthesis (Kinsella, 1972), and ensure sufficient fluidity of milk fat for efficient secretion from mammary cells (Kinsella, 1970). In contrast to non-ruminants, there is limited desaturase

activity in the liver of ruminants. Considerable desaturation of stearic acid occurs in the lactating ruminant mammary gland (Annison et al., 1967; Boyd et al., 1965; Laurysens et al., 1961), whereas mammary microsomes from non-lactating cows possessed little desaturase activity (Kinsella, 1972). Desaturase activity in the mammary gland converted 18:0 to *cis*9-18:1, the primary MUFA in milk fat. A significant proportion of the oleic acid in bovine milk is synthesized from stearic acid within the mammary gland (Kinsella, 1970).

Effects of dietary fat on milk fat composition

Up to about 1945, it had been a general perception among cattle nutritionists that milk composition, including that of fatty acids, could not be altered by diet (Van Soest, 1994). Boussingault (1845, cited by Van Soest, 1994) observed low milk fat when he fed cows a diet low in fat and fiber. This discovery led to the present understanding of the effects of diet on milk composition. Milk fat depression (MFD), a decreased milk fat percentage and milk fat yield, typically occurs when dairy cows are fed a low-fiber, high-grain diet or diets containing UFA or oils. Early explanations of MFD involved shortage theories, which consider the depression to be a consequence of a shortage of lipid precursors, including dietary fat, ruminal acetate, or β -hydroxybutyrate, to the mammary gland (Davis and Brown, 1970; Van Soest, 1963). Suppression of body fat mobilization by insulin may be involved in MFD (McClymount and Vallance, 1962). Enhanced insulin secretion in response to gluconeogenesis from propionate or absorption of glucose via excess dietary starch stimulates lipogenesis in adipose tissue and reduces release of fatty acids for uptake by the mammary gland. However, some evidence indicates that enhanced uptake of lipid precursors by adipose tissue during MFD may be a consequence rather than a cause of reduced mammary gland use of lipogenic precursors (Griinari et al., 1998). In addition, direct inhibition of mammary gland synthesis of milk fat by methylmalonic acid may be involved (Frobish and Davis, 1977). However, the role of methylmalonic acid, synthesized from propionate during a deficiency of vitamin B₁₂, in MFD was not supported by data showing no *in vitro* inhibition of mammary gland fatty acid synthesis (Croom et al., 1981). It is apparent that all these explanations are inadequate to account for MFD.

Supplemental dietary fat also can increase the concentration of fat in milk. The extent to which dietary fat will alter milk fat and milk fat composition depends on its effects on biohydrogenation, microbial fatty acid synthesis, and *de novo* synthesis of fatty acids in the mammary gland. This in turn depends on the physical form, fatty acid composition, and amount of dietary fat. For instance, moderately saturated fats tend to increase milk fat concentration (Sutton, 1989). It is generally believed that the depressing effects of UFA or oils rich in UFA must be transformed by the microbes in the rumen to produce an intermediate(s) that affects both blood lipid supply and the *de novo* fatty acid synthesis in the mammary gland. When McDonald and Scott (1977) fed protected lipid supplements containing linseed oil and safflower oil, milk fat contained in excess of 30% 18:2 and 20% 18:3, rather than the 3% and 1% of these fatty acids in milk from control cows.

Trans isomers of fatty acids are gaining more and more attention due to their inhibitory effect on milk fat synthesis in lactating ruminants and mice. It was suggested nearly 30 years ago that *trans* fatty acids might be responsible for the low-milk-fat syndrome (Davis and Brown, 1970). *Trans* isomers of oleic and linoleic acids are produced as by-products of the biohydrogenation process in the rumen. It was reported that dietary as well as ruminally derived *trans* fatty acids caused milk fat depression (Wonsil et al., 1994). Baughman (1995) demonstrated that *trans*-18:1 [elaidic acid or *trans*-vaccenic acid (TVA)] decreased lipid accumulation in mammary cell cultures. In vivo, conjugated linoleic acid (CLA) mixtures were shown to reduce de novo synthesis of saturated medium-chain fatty acids and desaturation of 18:0 in the mammary gland of cows (Lor and Herbein, 1998). Thus, relatively small quantities of supplemental TVA or CLA resulted in a significant decrease in fatty acid synthesis and desaturation in vitro or in vivo.

The regulatory mechanisms through which *cis* and *trans* isomers of 18-carbon fatty acids influence the rates of de novo fatty acid synthesis and desaturation remain unknown. Various theories have been set forth to elucidate the mechanism. The suggestion that *trans* fatty acids depressed milk fat synthesis due to their inability to enter the mammary gland for normal utilization (Rindsig and Schultz, 1974) was contradicted by several observations that *trans* isomers were readily taken up by bovine and mouse mammary tissue (Teter et al., 1990; Selner et al., 1980). It also was suggested (Hamosh and Clary, 1970) that *trans* fatty acids regulate the enzyme lipoprotein lipase, thus reducing uptake of fatty acids by mammary tissue.

Several investigators postulated that *trans* fatty acids inhibited acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD) (a critical enzyme in the biosynthesis of UFA), which introduces a *cis* double bond in the Δ^9 position (Hagemester, 1990). It was found that both CLA and TVA inhibited fatty acid synthase (FAS) activity in mouse mammary cell cultures (Jayan et al., 1998). Interestingly, CLA inhibited enzyme activity and mRNA abundance of SCD; whereas, TVA enhanced SCD enzyme activity and mRNA abundance. In the second part of their work with bovine mammary cell cultures, Jayan and Herbein (1999) observed similar effects of TVA and CLA on FAS enzyme activity and mRNA abundance, but both TVA and CLA enhanced SCD enzyme activity and mRNA abundance. Thus, CLA regulation of mammary SCD transcription may differ for nonruminant (mouse) and ruminant (bovine) species. Therefore, dietary fatty acids may modulate milk fatty acid composition by regulating the enzyme activity and mRNA abundance of enzymes involved in endogenous fat synthesis within the mammary gland.

Biosynthesis of saturated fatty acids

De novo fatty acid synthesis

De novo fatty acid synthesis occurs in the cytosol, and involves two major steps. The initial step is the ATP- and bicarbonate-dependent carboxylation of acetyl-

CoA to form malonyl-CoA, catalyzed by ACC. The second step is the conversion of acetyl-CoA and malonyl-CoA to palmitate, catalyzed by FAS. FAS uses NADPH as the reducing equivalent to synthesize palmitate.

The reaction catalyzed by ACC takes place in two steps: (1) carboxylation of biotin and (2) transfer of the carboxyl to acetyl-CoA to form malonyl-CoA. ACC is a multienzyme protein, containing a variable number of identical subunits, each containing biotin, biotin carboxylase, biotin carboxyl carrier protein, and transcarboxylase, as well as a regulatory allosteric site. This polymer can be dissociated into inactive protomers. Mammalian cells have two forms of ACC, which have been identified and play a critical role in controlling the amounts of fatty acids in cells (Kim, 1997). The enzyme that is involved in the synthesis of long-chain fatty acids has been designated ACC- α . The other isoform has been named ACC- β and it may be involved in the regulation of mitochondrial β -oxidation of fatty acids (Widmer et al., 1996).

Mammalian FAS is a multi-enzyme complex that may not be subdivided without loss of activity, and the acyl carrier protein (ACP) is part of this complex. The aggregation of all the enzymes of FAS into one multienzyme functional unit offers great efficiency and freedom from interference by competing reactions (Murray et al., 1997). Another advantage of a single multienzyme polypeptide is that synthesis of all enzymes in the complex is coordinated, since it is encoded by a single gene.

The FAS complex enzyme is a dimer, and only the dimer is active. In mammals, each monomer is identical, consisting of one polypeptide chain containing all seven enzyme activities of FAS and an ACP with a 4'-phosphopantetheine -SH group. In close proximity is another thiol of a cysteine residue of 3-ketoacyl synthase (condensing enzyme) of the other monomer. Therefore, palmitate is synthesized in seven sequential enzymatic reactions with intermediates attached to ACP. Initially, acetyl-ACP and malonyl-ACP are formed, catalyzed by acetyl transacylase and malonyl transacylase, respectively. Then the condensation of acetyl-ACP with malonyl-ACP forms acetoacetyl-ACP, catalyzed by acyl-malonyl-ACP condensing enzyme. Acetoacetyl-ACP is next reduced to D-3-hydroxybutyryl-ACP, catalyzed by β -ketoacyl-ACP reductase using NADPH as the reducing agent. Then D-3-hydroxybutyryl-ACP is dehydrated to form crotonyl-ACP, catalyzed by 3-hydroxyacyl-ACP dehydratase. The final step in the cycle reduces crotonyl-ACP to butyryl-ACP, catalyzed by enoyl-ACP reductase with NADPH as the same reducing agent. This sequence of reactions is repeated 6 more times, a new malonyl residue being incorporated during each cycle, until palmityl-ACP has been assembled. In the liver and adipose tissue, the final product of de novo synthesis is palmitate, liberated from the enzyme complex by thioesterase I. However, the cytosol of mammary epithelial cells of nonruminants contains a separate medium-chain acylthioester hydrolase, thioesterase II. Thioesterase II terminates fatty acid synthesis after growth to 8 to 14 carbons, resulting in the de novo synthesis of medium-chain fatty acids (Safford et al., 1987; Naggert et al., 1988). In ruminant mammary gland, this enzyme is part of the fatty acid synthase

complex. Bovine milk fat contains short-chain fatty acids. Butyryl-CoA and hexanoyl-CoA are synthesized by mammary gland FAS via the malonyl-CoA pathway (Hansen and Knudsen, 1980). Butyryl-CoA also originates from the conversion of β -hydroxybutyrate taken up by the lactating mammary gland of ruminants.

In nonruminants, acetyl-CoA is formed from glucose via the oxidation of pyruvate within the mitochondria. However, acetyl-CoA does not diffuse readily into the cytosol for fatty acid synthesis. It has to condense with oxaloacetate to form citrate, which is translocated into cytosol where it is cleaved to acetyl-CoA and oxaloacetate catalyzed by ATP-citrate lyase. The acetyl-CoA is then available for malonyl-CoA formation and synthesis to palmitate. The resulting oxaloacetate can form malate via NADH-linked malate dehydrogenase, and malate can in turn be converted into pyruvate via malic enzyme with the generation of NADPH. The NADPH becomes available for lipogenesis. There is little ATP-citrate lyase in ruminants, however. Therefore the main source of acetyl-CoA is from acetate, not glucose. Ruminants also have only negligible cytosolic NADP-dependent malic enzyme. As a result, the sources of NADPH for adult ruminants are the pentose phosphate cycle and the NADPH-dependent isocitrate dehydrogenase reaction. Since dietarily derived glucose is scarce in ruminant metabolism, the lack of cytosolic ATP-citrate lyase and malic enzyme in ruminants may be the mechanisms for glucose conservation for its most essential functions such as supplying energy for brain tissue and lactose synthesis in the mammary gland. The pathways of fatty acid synthesis in ruminants are shown in Figure 2.2.

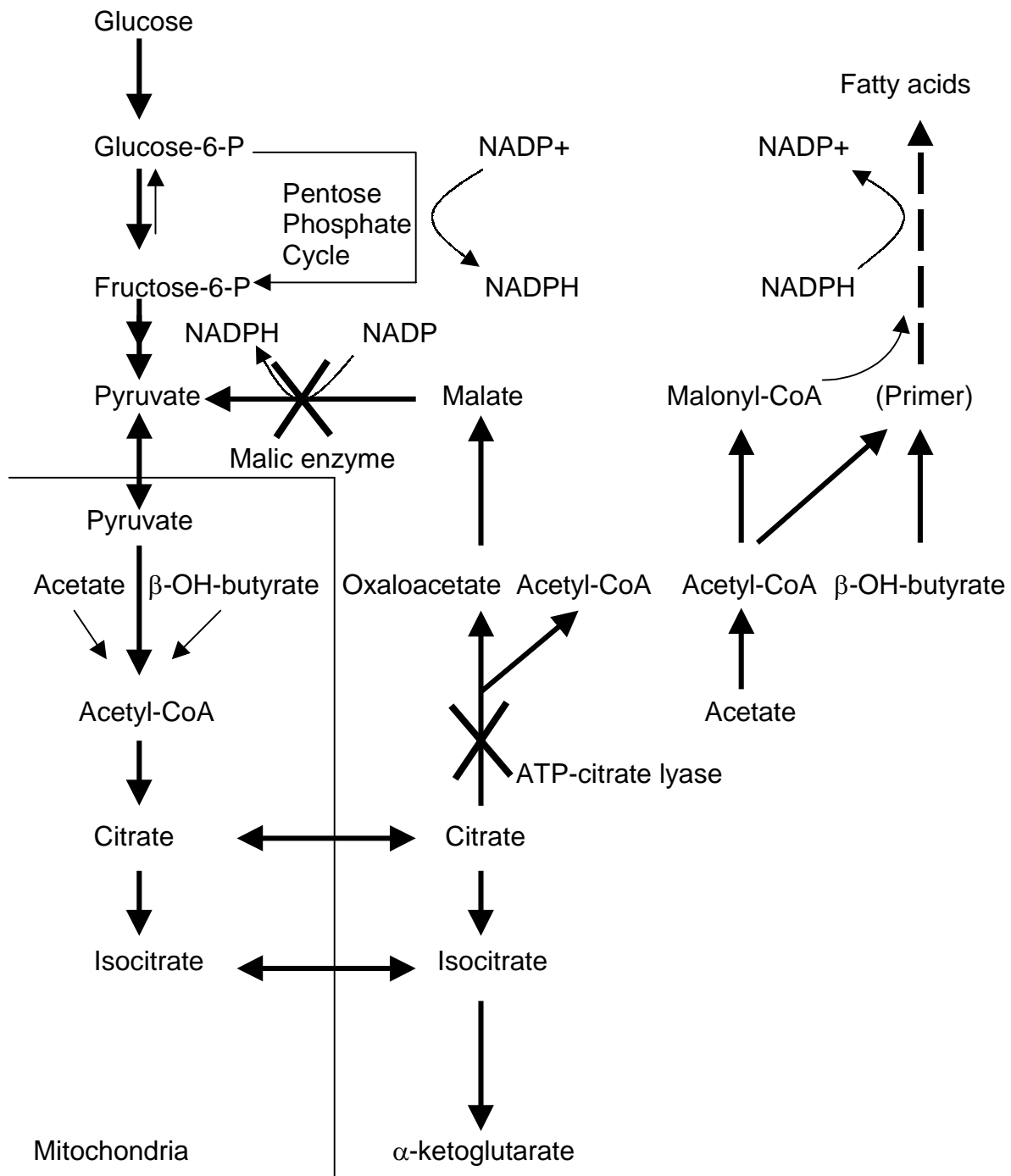


Figure 2.2 Pathways for fatty acid biosynthesis. (modified from Bauman and Davis, 1975).

Regulation of de novo fatty acid synthesis

Fatty acid metabolism is stringently controlled so that synthesis and degradation are highly responsive to physiological needs. Several regulatory strategies control fatty acid synthesis. These include substrate availability, regulation of key enzymes by short-term mechanisms, and control of the concentration of key enzymes (Goodridge, 1986). Factors regulating activities of lipogenic enzymes include nutritional (Clarke and Jump, 1996), hormonal (Vernon et al., 1991), and developmental conditions (Eritani et al., 1993). Enzyme activity is controlled by short-term systems that affect enzyme catalytic efficiency, and by long-term systems that control the quantity of active enzyme.

Short-term control: Covalent modification

Enzymes that have low activity relative to other members of the pathway are frequently considered as potentially rate limiting and are key control points for that pathway. The catalytic capacity of ACC was very low compared with that of FAS (Numa and Tanabe, 1984), and this suggested ACC was the primary rate-limiting enzyme controlling rate of fatty acid synthesis. Short-term or acute control by phosphorylation and dephosphorylation of ACC, a covalent modification, affects the catalytic efficiency. ACC is switched off by phosphorylation. Several hormones have been shown to control fatty acid synthesis by acute control mechanisms. Witters et al. (1979) reported that treatment of isolated hepatocytes or adipocytes with glucagon resulted in phosphorylation and inactivation of ACC, and phosphorylation of ACC by glucagon is possibly via activation of AMP-stimulated protein kinase (Hardie, 1992). Insulin, in contrast, increased fatty acid synthesis in liver and adipose tissue. However, insulin may not play a significant regulatory role in de novo synthesis in the lactating mammary gland. In the case of MFD discussed above, the glucogenic-insulin theory (Jenny et al., 1974) proposes that enhanced insulin secretion in response to gluconeogenesis from propionate or absorption of glucose via excess dietary starch stimulates lipogenesis in adipose tissue and reduces release of fatty acids for uptake by the mammary gland. However, some evidence indicates that enhanced uptake of lipid precursors by adipose tissue during MFD may be a consequence rather than a cause of reduced mammary gland use of lipogenic precursors (Griinari et al., 1998).

Unlike ACC, FAS is not subject to allosteric regulation. Rather, FAS is regulated mainly by controlling the rate of transcription.

Long-term control: Regulation of gene expression

During the recent decade or so, numerous studies have revealed that many nutrients and vitamins seem to act essentially in the same way as steroidal hormones, i.e., by binding to soluble intranuclear receptors. The ligand-receptor complexes then bind to promoters of regulated genes, thereby affecting their rate of transcription (Gustafsson, 1998). The expression of lipogenic genes is regulated by hormones and dietary factors, including fatty acids.

There are two distinct genes encoding ACC, ACC- α and ACC- β . (Luo et al., 1989; Ha et al., 1996). ACC- α is expressed in all cell types but demonstrates elevated

expression and is the major form in the adipose tissue and liver, and in the mammary gland during lactation (Lopez-Casillas et al., 1991; Winz et al., 1994; Abu-Elheiga et al., 1995). ACC- β is the major form in heart and skeletal muscle, where it is implicated in the regulation of fatty acid β -oxidation in mitochondria (Widmer et al., 1996). Furthermore, transcription of the ACC- α gene is initiated from two promoters, promoter I (PI) and promoter II (PII), resulting in transcripts with heterogeneity in the 5' untranslated region (Luo et al., 1989).

Hormonal control FAS mRNA abundance in hepatocyte cultures was stimulated by insulin but inhibited by glucagon through gene transcription (Goodridge, 1986). It also was reported that increased activities of hepatic ACC and FAS in mice fed a fat-free, high-carbohydrate diet was primarily due to increased transcription from their respective genes (Clarke et al., 1990). Fatty acid synthesis in the rat is changed by thyroid status in a tissue-specific manner (Blennemann et al., 1992). Experiments from the same lab indicated that this regulation occurs at the level of ACC and FAS mRNA levels (Blennemann et al., 1995). Thyroid hormone regulates the activity of the ACC PI promoter to influence fatty acid synthesis in a tissue-specific manner (Huang and Freake, 1998).

Nutritional control Hillgartner et al. (1996) reported that feeding previously starved chicks a high-carbohydrate, low-fat diet stimulated a 9-fold increase in both the rate of synthesis of ACC and abundance of its mRNA in liver. The transcriptional activity of ACC demonstrated that nutritional control of the abundance of ACC mRNA in the chicken is mediated by changes in the rate of transcription of the ACC gene. Results also showed that the nutritional control was liver-specific, since the abundance of ACC mRNA in heart, pectoral muscle, kidney and brain was not affected.

Types and amounts of dietary fatty acids can be important factors modulating de novo fatty acid synthesis. The ability of different fatty acids to inhibit fatty acid synthesis varies and is tissue-specific (Souza and Williamson, 1993). PUFA inhibited mammary gland fatty acid synthesis to the greatest extent, followed by MUFA and SFA (Souza and Williamson, 1993). PUFA inhibited fatty acid synthesis in the liver; however, diets rich in MCFA increased hepatic fatty acid synthesis. Dietary fatty acids act transcriptionally or post-transcriptionally on genes encoding lipogenic enzymes. PUFA have been shown to suppress the expression of several lipogenic genes in the liver, including transcription of FAS (Blake and Clarke, 1990). Diets rich in long-chain triglycerides suppress the induction of ACC and FAS in the adipose tissue and liver of weaned rats, primarily at the transcriptional level (Girard et al., 1994).

Biosynthesis of unsaturated fatty acids

The role of stearoyl-CoA desaturase

Desaturation of a fatty acid involves the enzymatic removal of hydrogen from a methylene group in an acyl chain, an energy-demanding step that requires an

activated oxygen intermediate (Shanklin and Cahoon, 1998). Fatty acid desaturases exist in all organisms, except some bacteria such as *Escherichia coli*.

Free fatty acids must be esterified to acyl carrier protein (ACP), coenzyme A, or lipids prior to desaturation by one of three types of fatty acid desaturases: acyl-ACP, acyl-CoA, or acyl-lipid desaturases (Los and Murata, 1998). Acyl-lipid desaturases are membrane bound enzymes and are present in plants and cyanobacteria (Murata et al., 1992). Acyl-ACP desaturases are soluble and present in the plastids of plant cells (Bloomfield and Bloch, 1960). Acyl-CoA desaturases also belong to the membrane class, but are present in animal, yeast, and fungal cells (Macartney et al., 1994).

Stearoyl-CoA desaturase (SCD), an acyl-CoA desaturase, is an iron-containing microsomal enzyme that catalyzes the critical committed step in the biosynthesis of MUFA by introducing the first *cis* double bond in the Δ^9 position. The desaturase system involves three enzyme components: cytochrome b_5 , NADH-cytochrome b_5 reductase, and desaturase. Only the terminal desaturase's activity is sensitive to changes in diet, hormonal balance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds (Ntambi, 1995). The two electrons needed are transported through an electron-transport system that is composed of cytochrome b_5 and NADH-dependent cytochrome b_5 reductase, as illustrated in Figure 2.3.

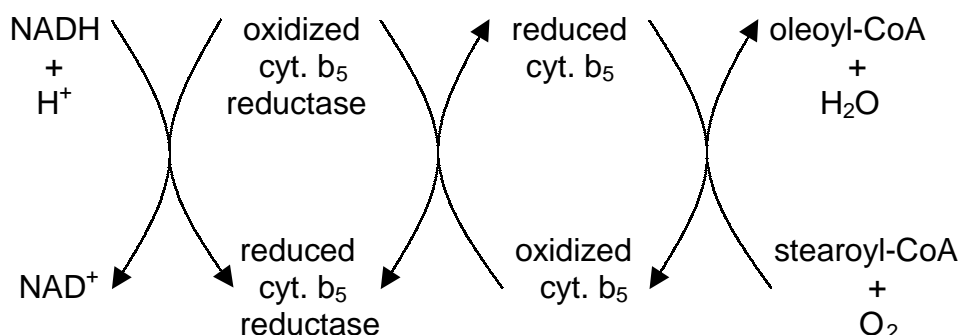


Figure 2.3 The pathway for electron transfer in the desaturation of fatty acids by SCD. (adapted from Ntambi, 1995)

Regulation of stearoyl-CoA desaturase

Substrate specificity of SCD

SCD catalyzes the Δ^9 -*cis* desaturation of a number of methylene-interrupted fatty acyl-CoA substrates. However, the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (Enouch et al., 1976). Palmitoleate (*cis*9-16:1) and oleate (*cis*9-18:1) are the major constituents of membrane phospholipids and triglyceride stores in adipose tissue (Kasturi and Joshi, 1982). Membrane fluidity is directly affected by the balance between stearic and oleic acids, and the changes in this ratio have been

implicated in disease states including diabetes, obesity, hypertension, cancer, and neurological, vascular and heart diseases (Ntambi, 1999).

Acyl-CoAs of some UFA also can be the substrates for SCD. Pollard et al. (1980) reported desaturation of a broad range of *trans* monoenes to *cis,trans*-18:2 derivatives by isolated rat liver microsomes. Interestingly, desaturation of *trans*11-18:1, the predominant *trans* monoene in bovine milk fat, results in formation of *cis*9,*trans*11-18:2, a CLA isomer that has drawn much attention due to its positive health effects. Conversion of 11% of dietary *trans*11-18:1 to *cis*9,*trans*11-18:2 in non-lactating mice was reported (Santora et al, 2000). In lactating mice, dietary TVA increased the *cis*9,*trans*11-18:2 content in blood plasma lipids, mammary gland, liver, and carcass, and increased SCD activity was observed in the mammary gland (Loor et al., 1999).

Fatty acid regulation of SCD genes

The genes coding for rodent SCD have been cloned (Ntambi et al., 1988; Kaestner et al., 1989), and designated SCD1 and SCD2. SCD1 is expressed in adipose tissue under normal physiological conditions. SCD2 is expressed in brain, but not in adipocytes or liver. By contrast, a single SCD gene is present within the ovine genome (Ward et al., 1998).

Very low levels of hepatic SCD1 expression are observed in rodents under normal dietary conditions. A fat-free diet induces its expression markedly, indicating that hepatic SCD1 is suppressed by a fat component in the normal diet (Ntambi, 1995). Ample evidence has indicated that PUFA suppress the expression of SCD. Uchiyama et al. (1967) observed depression of desaturation by linoleic and arachidonic acids, and oleic acid to a lesser extent in vitro. Landschulz et al. (1994) reported depression of SCD1 mRNA increased with degree of unsaturation in rat liver both in vitro and in vivo, and the depression was due to inhibition of SCD1 gene transcription. In mouse adipocyte cultures, Sessler et al. (1996) observed a decrease in SCD1 mRNA in response to arachidonic, linoleic, and linolenic acids, but not oleic acid or stearic acid. A response region (*cis*-acting element) to PUFA in the SCD1 promoter region in mouse hepatic cells has been identified (Waters et al., 1997). This region showed similar binding of nuclear proteins from adipocyte nuclear extracts, indicating that a common transcriptional mechanism may exist in liver and adipose tissue for inhibition of desaturase by PUFA.

It is evident that regulation of SCD gene by PUFA is well studied in liver and adipose tissue, but not in the mammary tissue. It was shown that *cis* and *trans* isomers of 18:1 and 18:2 regulated SCD enzyme activity and mRNA abundance in both mouse (Jayan et al., 1998) and bovine mammary epithelial cells (Jayan and Herbein, 1999). However, it still remains unclear whether these fatty acids act on transcription or at the post-transcriptional level on SCD gene expression.

Regulatory roles of fatty acids and regulatory mechanisms

Fatty acids affect not only expressions of lipogenic genes like ACC, FAS, and SCD, but also those involved in glycolysis, glucose transport, inflammation, early gene expression, and vascular cell adhesion molecules (Simopoulos, 1996). The mechanisms by which fatty acids regulate lipogenic gene expression will be the focus here. Sessler et al. (1996) reported that the half-life of SCD1 mRNA in mature adipocytes was 67% lower when cells were treated with arachidonic acid. PUFA, on the other hand, directly acted on transcription of SCD1 gene (Clarke and Jump, 1994).

One mechanism by which PUFA regulate gene transcription focuses on the idea that a *cis*-acting PUFA responsive element (PUFA-RE, a DNA sequence) is located in the promoter region of the PUFA-regulated genes. A transcription factor (putative PUFA-binding protein: BP) could form a PUFA-BP complex and bind to a PUFA-RE to block or enhance transcription (Sessler and Ntambi, 1998). A 60-bp PUFA-RE region in the promoters of SCD1 and SCD2 has been localized and nuclear factor binding to this element also has been demonstrated (Waters et al., 1997).

Another mechanism by which fatty acids modulate gene expression is the recent discovery that fatty acids regulate gene transcription through their interactions with nuclear receptors, such as peroxisome proliferator-activated receptors (PPAR). PPAR belong to orphan receptors, which lack known ligands, but are involved in a large number of physiologic and pathophysiologic processes (Kastner et al., 1995). Three distinct subtypes of PPAR (α , δ , and γ) have been identified. PPAR α is preferentially expressed in liver, and it regulates the expression of genes encoding enzymes in the peroxisomal β -oxidation pathway (Gervois et al., 2000). PPAR γ is expressed mainly in adipose tissue and modulates lipid homeostasis. PPAR δ is the most widely expressed isoform.

PPAR have a large ligand binding domain that can bind many different compounds, which explains why PPAR can be activated by a broad range of compounds including fatty acids (Gustafsson, 1998). PPAR do not function as homodimers or monomers, but strictly depend on retinoid X receptors (RXR) as DNA-binding partners (Lemberger et al., 1996). PUFA bind to the PPAR/RXR heterodimer and this complex through the DNA-binding domain binds to PPAR response elements in the promoters of target genes, activating or repressing the transcription of regulated genes.

More recent research indicates that fatty acids may modulate gene expression through another transcription factor, sterol regulatory element binding proteins (SREBP). SREBP synthesized as precursors, bound to the endoplasmic reticulum and nuclear envelope, are released into the nucleus upon activation (Yahagi et al., 1999). Three SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2, have been identified and characterized (Yahagi et al., 1999). SREBP-1c is the predominant form in the liver, and it primarily regulates fatty acid synthesis in the liver. SREBP-

1c has been reported to regulate lipogenic gene expression by binding to the sterol response element (SRE) of ACC and FAS genes (Sul and Wang, 1998). Xu et al. (2000) reported that PUFA regulate lipogenic genes by inhibiting SREBP-1 nuclear localization and by suppressing SREBP-1 synthesis through enhanced mRNA decay. It was found that both SREBP-1a and SREBP-2 enhanced mouse SCD2 gene transcription (Tabor et al., 1998). SREBP-1a and SREBP-2 also regulated SCD1 gene transcription, and both SCD1 and SCD2 contained a novel SRE that is necessary for their sterol-dependent transcription. Maximal transcriptional repression of SCD2 in response to PUFA supplementation depends upon the SRE (Tabor et al., 1999).

In summary, fatty acids have a large regulatory network through which they modulate gene transcription. Regulation can be via direct binding to the promoter region of regulated genes, through interactions with PPAR, or by regulating the abundance of nuclear SREBP.

Fatty acids and human health

Negative health implications

High SFA intake has been implicated in a number of human health problems, such as coronary heart disease (CHD), and the main concern about excess SFA intake is on their potential role in raising blood cholesterol. Cholesterol travels through blood associated with lipoproteins. Too much blood cholesterol slowly deposited on the inner wall of arteries may lead to atherosclerosis, which is regarded as a principal cause for CHD. Blood cholesterol reflects the amount of three major classes of lipoproteins: very-low-density lipoproteins (VLDL); low-density lipoproteins (LDL), which contains most of the cholesterol found in the blood; and HDL. Total serum cholesterol does not predict CHD well, since it has been shown that the HDL-cholesterol is the 'good' cholesterol, being strongly and inversely related to CHD risk (Stampfer et al., 1991). In contrast, LDL-cholesterol seems to be the culprit and is associated with cholesterol deposits on artery walls (NRC, 1989). However, saturated fats and dietary sources of saturated fat vary in their effect on LDL-cholesterol levels. Butter and other dairy fats (rich in myristic acid (14:0)) strongly increase LDL levels, beef fat (containing 16:0 and 18:0) increases LDL levels to a lesser degree, and cocoa butter (mainly stearic acid) enhances LDL levels only slightly (Willett, 1994). When stearic acid was provided at a level three times higher than that in the baseline diet, stearic acid reduced LDL-cholesterol but did not alter the ratio of LDL- to HDL-cholesterol (Aro et al., 1997). It is apparent that all SFA are not equal in their cholesterol-raising effects and it appears that stearic acid may be neutral in its effect on serum LDL-cholesterol level.

It was reported that substitution of monounsaturated for saturated fats decreased plasma levels of total and LDL-cholesterol while preserving HDL-cholesterol levels (Grundy et al., 1988). It also was observed that replacing saturated fats with *cis* isomers of monounsaturated and polyunsaturated fats was more effective in

preventing the incidence of CHD than reducing the overall fat intake (Hu et al., 1997). A reduced risk of CHD was associated with fish consumption, both in a prospective study and a randomized trial among patients with a previous infarction (Willett, 1994). Fish is a rich source of n-3 fatty acids (with a double bond on the third carbon atom from the methyl end) and it was therefore suggested that n-3 fatty acids may promote a protective effect against the development of coronary artery disease.

Trans fatty acids have drawn much attention due to their association with the risk of CHD. *Trans*-vaccenic acid is one of the intermediates during hydrogenation of UFA in the rumen. Significant amounts of *trans*-vaccenic acid are either deposited in tissues or secreted into the milk. However, most dietary *trans* fatty acids in human foods are produced when liquid vegetable oils are hydrogenated to form vegetable shortenings and margarine, accounting for 3 to 6% of the daily fat intake in the U.S. population (Enig et al., 1990). Of the *trans* fatty acids from partial hydrogenation of vegetable oils, elaidic acid (*trans*9-18:1) is the most abundant.

Substantial data support the notion that the *trans* fatty acids from partially hydrogenated vegetable oils adversely affect the risk of CHD. Mensink and Katan (1990) compared a diet containing 11% of calories from *trans* fatty acids with a similar diet containing oleic acid. The *trans* fatty acid diet increased LDL-cholesterol and reduced HDL-cholesterol. Lipoprotein(a), another factor positively associated with CHD risk, also was increased by the *trans* fatty acid diet. Epidemiologic findings also supported the connection between *trans* fatty acids and the risk of CHD. In a study of 89,095 female registered nurses, 431 new cases of CHD were found during an 8-year follow-up (Willett et al., 1993). Intake of *trans* isomers was directly related to risk of CHD after adjustment for age and total energy intake.

In examining the effects of *trans* fatty acids, it is very important to note that different *trans* fatty acids may have specific physiological effects. In a study with human subjects where elaidic acid at 5.5% of energy intake was specifically exchanged for *cis*-18:1, 16:0, or 12:0 + 14:0, the elaidic acid-rich diet significantly elevated total cholesterol and LDL-cholesterol relative to the 16:0- and 18:1-rich fats and uniquely depressed HDL-cholesterol relative to all of the fats tested (Sundram et al., 1997). Elaidic acid also elevated lipoprotein(a) values relative to all dietary treatments. *Trans*-vaccenic acid, on the other hand, is still not known to bear negative effects on human health.

The influence of *trans* fats on human cholesterol levels may be a function of linoleic acid. Kritchevsky (1997) compared nine studies, in four of which an average increase in serum cholesterol of $9.9 \pm 1.6\%$ (SEM) was associated with the ratio of *trans* fat to 18:2 being greater than 2. In the other five, where the ratio was below 2.0, cholesterol levels rose by $2.1 \pm 0.4\%$ (SEM). This is consistent with the observation that *trans* fatty acids impaired desaturation and elongation of 18:2 to 20:4 (Sugano and Ikeda, 1996), and the detrimental effect of *trans* fatty acids disappeared when dietary linoleic acid was sufficiently supplemented. On the other

hand, elaidic acid, but not *trans*-vaccenic acid, was preferentially incorporated in phosphatidylethanolamine and phosphatidylcholine, where elaidic acid may affect membrane bound desaturation and elongation enzymes (Woldseth et al., 1998).

Positive health implications

Recent research has shown that milk fat contains a number of components that are beneficial to human health. Of these, CLA have been shown to have many physiological effects. CLA is a collective term for positional and geometric isomers of linoleic acid with two conjugated double bonds (Parodi, 1994). The two conjugated double bonds can be present at the following positions: 8 & 10; 9 & 11; 10 & 12; and 11 & 13, with each double bond in either *cis* or *trans* configuration. CLA isomers are usually found in milk and meat products. Ruminant fats contain much higher levels of CLA than lipids from non-ruminants (Chin et al., 1992). The higher proportion of *cis*9,*trans*11-18:2 in milk and tissues from ruminants is due to a specific geometric and positional isomerization of linoleic acid by ruminal bacteria (Kepler et al., 1966). *Cis*9,*trans*11-18:2 also could be produced endogenously from *trans*-vaccenic acid via SCD (Parodi, 1994).

Studies in vitro and in experimental animals showed that *cis*9,*trans*11-18:2 is anticarcinogenic. Shultz et al. (1992) observed that CLA inhibited human cancer cell growth in culture in a dose-dependent manner. *Cis*9,*trans*11-18:2 was shown to have anticarcinogenic activity against induced skin cancer and forestomach neoplasia in mice (Ha et al., 1990).

Recent research showed evidence regarding the effect of dietary CLA on plasma lipoproteins and aortic atherosclerosis. Lee et al. (1994) reported that the supplementation of 0.5 g CLA per day for 22 weeks to rabbits fed an atherogenic diet significantly reduced plasma triglycerides, plasma LDL-cholesterol, and the ratio of LDL-cholesterol to HDL-cholesterol. CLA feeding also resulted in fewer aortic fatty lesions. Nevertheless, there is still a paucity of information on the relationship between CLA and lipoprotein profile.

Several studies have shown that CLA may regulate body composition in several experimental models, including mice, rats, pigs, and chickens. Park and coworkers (1997) reported feeding CLA (0.5%) to mice resulted in a 60% reduction of body fat and a 5% increase in lean body mass compared to a control group fed corn oil (5.5%). Interestingly, Park et al. (1999) demonstrated that the *trans*10,*cis*12-18:2 may be the active isomer modulating body composition. Similarly, Looor et al. (2000) observed that 1% pure *trans*10,*cis*12-18:2 in the diet of mice significantly reduced fat and increased protein content (% of body weight) in the carcass. However, the isomer reduced food intake in both studies. The extent to which reduced food intake (in combination with *trans*10,*cis*12-18:2) influenced body composition has not been determined.

In summary, some of the saturated fatty acids in milk fat are detrimental to health, whereas, unsaturated fatty acids may be beneficial. Elaidic acid from partially hydrogenated vegetable oil, but not *trans*-vaccenic acid from dairy products, may

be responsible for an increase of CHD. CLA have newly recognized biological activities. Therefore, it is important to produce dairy foods with a balance of various fatty acids, preferably with less saturated fatty acids, more unsaturated fatty acids, and CLA.

In Vitro model cell lines to study gene regulation in the mammary gland

Cell culture provides a useful, economical, and relatively controlled tool for studying cellular activity in vitro. Primary cell cultures are derived from animal tissues. However, they have a finite life span, growing slowly after a few generations and ceasing growth finally. Cell strains or permanent cell lines can grow indefinitely in culture and are derived either from tumor cells or from transformed cultured cells.

COMMA-D and COMMA-D/MME cell lines

COMMA-D is a mouse mammary epithelial cell line, which was derived from normal mammary gland tissue of BALB/c mice in the middle of pregnancy. (Danielson et al., 1984). COMMA-D retained mammary gland-specific morphological and functional differentiation in vitro. They exhibited cuboidal epithelial-like morphology when cultured on plastic; monolayers also formed when cells were in close contact to each other. Therefore, the COMMA-D cell line provides a way to study mouse mammary gland biology and differentiation in vitro.

The COMMA-D/MME cell line is a subpopulation of the COMMA/D cell line. Skaar and Baumrucker (1993) investigated the regulation of insulin-like growth factor (IGF) binding protein secretion by mammogenic and lactogenic hormones in COMMA-D/MME cells. IGF binding was studied in this cell line, which showed mitogenic responses to IGF (Hadsell et al., 1994). COMMA-D/MME were capable of partial and transient differentiation to synthesize milk lipids (Gibson and Baumrucker, 1996).

The Mac-T cell line

Mac-T is a bovine mammary epithelial cell line for study of bovine lactation. It was produced from primary bovine mammary alveolar cells (Mac-T) by stable transfection with SV-40 large T-antigen (Huynh et al., 1991). The cells showed the characteristic 'cobblestone' morphology of epithelial cells when grown on plastic substratum. They expressed β -casein mRNA and secreted casein proteins upon differentiation.

The Mac-T cell line has been useful in the study of intrinsic and extrinsic factors that regulate bovine mammary epithelial cell development, differentiation, and function. Rejman et al. (1992) identified several local factors associated with regulation of mammary epithelial cell proliferation using Mac-T cells. Romagnolo et al. (1994) studied the mechanisms by which IGF-I and insulin regulated IGF-I binding proteins (IGFBP) in Mac-T cells. Regulation of mammary microsomal fatty acyltransferase activities was investigated in Mac-T cells by Morand et al. (1998). It was observed that Mac-T cells displayed acyltransferase activities that were consistent with bovine mammary tissues, and the enzyme activity was both

stimulated by insulin and prolactin. Therefore, the Mac-T and the COMMA-D/MME cell lines are useful models for study of lactogenesis, fatty acid metabolism, and gene regulation in the mammary gland.

CHAPTER 3

DESATURATION OF *TRANS*-VACCENIC ACID TO *CIS*₉,*TRANS*₁₁ CONJUGATED LINOLEIC ACID AND THEIR EFFECTS ON ACTIVITIES OF LIPOGENIC ENZYMES IN LACTATING MOUSE TISSUES

ABSTRACT

The lactating mouse was used as a model to investigate conversion of dietary *trans*-vaccenic acid (*trans*_{11-18:1}) (TVA) to conjugated linoleic acid (*cis*₉,*trans*_{11-18:2}) (9/11CLA) and the effects of dietary TVA and CLA on activities of lipogenic enzymes in liver and mammary tissue. Lactating mice were fed limited amounts (9 g/d) of diets containing 3% linoleic acid (LA) or 2% LA plus 1% stearic acid (SA), TVA, or a CLA mixture during d 3 to 14 postpartum. The 9/11CLA content of liver and mammary tissue of mice fed LA or SA were barely detectable and was only 0.1% of total fatty acids in the carcass. In contrast, 9/11CLA concentrations in liver (0.55%) and carcass (0.60%) of mice fed TVA were similar to those in liver (0.51%) and carcass (0.69%) of mice fed CLA. Mammary tissue of TVA-fed mice, however, contained 0.50% 9/11CLA compared with 1.27% 9/11CLA for CLA-fed mice. In pups nursing TVA-fed dams, 9/11CLA accounted for 0.26% of liver fatty acids and 0.03% of carcass fatty acids, whereas 9/11CLA accounted for 0.76% and 0.56% of fatty acids in liver and carcass of pups nursing CLA-fed dams. An increase in SCD activity in mammary tissue was associated with feeding TVA to lactating mice, and an increase in SCD activity in liver of their pups also was observed. Dietary TVA did not affect activities for acetyl-CoA carboxylase (ACC) or fatty acid synthase in tissues of lactating dams or their suckling pups, but dietary CLA reduced mammary ACC activity and total fatty acid content of mammary tissue. Results indicated dietary TVA was desaturated to 9/11CLA in tissues of lactating mice, possibly via enhanced SCD activity, and dietary TVA can be used as a means to enhance the 9/11CLA content of tissues of their suckling pups.

INTRODUCTION

The CLA abbreviation is a collective term for positional and geometric isomers of linoleic acid with a pair of conjugated double bonds (Parodi, 1994). The *cis9,trans11-18:2* isomer (9/11CLA) was identified as the biological agent responsible for reduced tumor formation during in vitro and in vivo studies. Shultz et al. (1992) observed that CLA inhibited human MCF-7 breast cancer cell growth in culture in a dose-dependent manner, and 9/11CLA was shown to have anticarcinogenic activity against induced skin cancer and forestomach neoplasia in mice (Ha et al., 1990).

The 9/11CLA isomer is normally found in milk and meat products. Food lipids originating from ruminant animals (beef, lamb, and dairy) contain much higher levels of 9/11CLA than lipids from non-ruminants (Chin et al., 1992). The high proportion of 9/11CLA isomer in milk and tissues from ruminants is due to a specific geometric and positional isomerization of linoleic acid by ruminal bacteria (Kepler et al., 1966). Saturation of the *cis9* bond in 9/11CLA yields *trans11-18:1* (TVA), which is the prominent *trans* fatty acid in milk fat. Mean isomeric distribution of *trans-18:1* isomers extracted from 1,756 samples of milk fat indicated that TVA accounted for 43.2% of total *trans-18:1* (Molkentin, 1999). Thus, bovine milk provides TVA and 9/11CLA in the diets of humans.

More interestingly, 9/11CLA can be synthesized endogenously via stearoyl-CoA desaturase (SCD) from TVA. Pollard et al. (1980) reported that rat liver microsomal SCD desaturated several *trans* monoenoic acids to *cis, trans-18:2* derivatives. In that study exogenous TVA was desaturated to the greatest extent (62%) to 9/11CLA by isolated rat liver microsomes, whereas, *trans10-18:1* was not desaturated. In contrast, Holman and Mahfouz (1981) reported the rate of TVA desaturation was only about one-third of that for *trans* isomers of 4-, 6-, and 13-monenenes. Mahfouz et al. (1980b) also reported Δ^9 desaturation of *trans 18:1* isomers in rat liver microsomes, indicating action of SCD. In the above studies, SCD introduced a *cis* double bond at the Δ^9 position of *trans11-18:1*, resulting in *cis9,trans11-18:2*.

The liver may be one of several sites for desaturation of TVA in rodents. Growing mice converted 11% of dietary TVA and 51% of stored TVA to 9/11CLA (Santora et al., 2000). The authors suggested that the bioconversion took place in adipose tissue. In addition, Jayan (1998) observed that the concentration of 9/11CLA in mouse mammary cell cultures increased from 0 to 2.8% in response to TVA supplementation from 0 to 100 μ M. Therefore, it appears that mammary cells in vitro also can undergo desaturation of TVA to CLA.

Because SCD is required for conversion of TVA to 9/11CLA, it is important to know how TVA and CLA regulate SCD activity. In association with increased 9/11CLA content in response to TVA supplementation in the medium, there were increases in SCD enzyme activity and mRNA abundance in the cells (Jayan,

1998). In vivo, CLA infused abomasally decreased desaturation of fatty acids as indicated by an increased ratio of 18:0 to 18:1 (Chouinard et al., 1999a). However, SCD activity was not measured in that study. Apparently, there is a lack of information about SCD regulation by dietary TVA or CLA in vivo. Therefore, the present study was to investigate the extent to which dietary TVA may contribute to 9/11CLA in tissues of lactating mice and suckling pups. At the same time, effects of dietary TVA and CLA on activities for acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and SCD also were investigated.

MATERIALS AND METHODS

Animals

Female CD-1 mice were maintained and bred in the Dairy Science mouse colony at Virginia Tech. They were fed a Harlan Teklad (Harlan, Madison, WI) S-2335 mouse-breeder diet, which was nutritionally complete. The diet and water were provided for ad libitum consumption. Mice were housed in individual polypropylene cages in a temperature-controlled room (23°C) with a 12-h light and 12-h dark cycle. The experimental protocol was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1985).

Diets

The breeder diet was supplemented with 30 g linoleic acid (Cat. #: 18440; 65% purity; United States Biochemical Corp., Cleveland, OH) per kilogram diet (LA) or 20 g LA plus 10 g stearic acid (Code #: N-18-A; >99% purity; Nu-Check Prep, Elysian, MN), 10 g *trans*-vaccenic acid (Code #: U-49-A; >99% purity; Nu-Check Prep), or 10 g of a mixture of conjugated linoleic acids (Tonalin CLA90; 44% *cis*9,*trans*11-18:2 and 41% *trans*10,*cis*12-18:2; Natural Lipids, Norway) per kilogram of diet. Diets were designated LA (control), SA (stearic acid), TVA, or CLA.

Design

Dams were fed the LA diet during the first 2 d of lactation. On d 3, six lactating mice were randomly assigned to each dietary group and fed 9 grams of diet per day. Litters were standardized to six pups each on d 3. One dam in the CLA group lost all its pups, ceased lactating, and was removed. Lactating mice and their pups were sacrificed to obtain tissues on d 14.

Dams and pups were anesthetized with Metofane to obtain a blood sample by heart puncture, then sacrificed by cervical dislocation. Liver and mammary glands were recovered, weighed, placed in liquid nitrogen, and stored at -80°C until subsequent analyses. Head, tail, heart, skin, feet, lungs, and the gastrointestinal tract were then removed. What remained constituted the carcass, which was weighed and stored at -20°C. The carcasses were lyophilized (Dura-Top freeze dryer, FTS Systems, Inc., Stone Ridge, NY) and ground prior to fatty acid analysis. Pup carcasses within a litter were pooled for lyophilizing, grinding, and fatty acid analysis. Pups livers within a litter were also pooled for enzyme assays.

Fatty acid analysis

Lipids were extracted from liver, mammary tissue, and carcass with chloroform/methanol (2:1, vol/vol). Fatty acids in tissue lipids were directly methylated using 0.5 N NaOH in methanol, according to the base-catalyzed methylation of Park and Goins (1994). Undecenoate (Nu-Check Prep) was used as an internal standard for peak quantification in the gas chromatogram. Samples were injected by auto-sampler into a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Sunnyvale, CA).

Methyl esters of fatty acids were separated on a 30 m x 0.25 mm i.d. fused silica capillary column (SP-2380, Supelco, Inc., Bellefonte, PA).

Preparation of samples for ACC, FAS, and SCD enzyme assays

Tissues were suspended in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, and 1 mM glutathione) at a ratio of 1 gram of tissue to 3 mL of buffer. The tissue was homogenized with a Polytron[®] homogenizer (Type PCU-2; Switzerland). All centrifugations were performed at 2°C. The homogenate was centrifuged at 5,000 x g for 15 minutes to remove nuclei, cell debris, and the fat cake on the surface. The supernatant was transferred and centrifuged for 20 minutes at 20,000 x g to pellet mitochondria. The supernatant was transferred to an ultracentrifuge tube on ice and centrifuged at 105,000 x g for 60 minutes. The supernatant thus obtained was stored at -80°C until used for assay of ACC and FAS activity. The microsomal pellet was resuspended in 0.4 mL buffer (50 mM Tris-HCl buffer, pH 7.4, and 1 mM glutathione) and stored at -80°C until analysis for SCD.

Microsomal protein determination

Protein contents of the supernatant and microsomal pellet were determined using the spectrophotometric bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL). The micro-well plate protocol as described by the reagent manufacturer was used. Absorbance was read at 560 nm on an Automated Microplate Reader (EL311s; BIO-TEK Instruments, Inc., Winooski, VT). The protein content in each sample was calculated from a standard curve plotted using known concentrations of bovine serum albumin.

ACC assay

ACC activity was assayed by the bicarbonate fixation protocol (Gregolin et al., 1966), which follows the rate of acetyl-CoA-dependent fixation of [¹⁴C]-bicarbonate into acid-stable [¹⁴C] activity (malonyl-CoA). The samples were first pre-incubated at 37°C for 15 minutes to activate the enzyme. The pre-incubation mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM sodium citrate, 10 mM MgCl₂, 3.75 mM glutathione, and bovine serum albumin (0.75 mg/mL) in a total volume of 1 mL. Reaction was started by adding 0.4 mL of the pre-incubated sample (0.4 mg protein) to 0.4 mL of assay mixture. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM sodium citrate, 10 mM MgCl₂, 3.75 mM ATP, 0.125 mM acetyl-CoA, 3.75 mM glutathione, bovine serum albumin (0.75 mg/mL), 12.5 mM sodium bicarbonate, and 50,000 CPM of NaH¹⁴CO₃ (59 Ci/mol; Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The reaction was allowed to proceed for 20 minutes at 37°C, then terminated with 0.2 mL of 6 N HCl. Centrifugation at 15,000 x g for 2 minutes was used to eliminate the insoluble material. A 0.3 mL aliquot of the supernatant was transferred to a scintillation vial, then taken to dryness in a 70°C water bath using continuous nitrogen flow to completely remove un-reacted H₂¹⁴CO₃. After addition of 0.3 mL distilled water and 10 mL of scintillation fluid, the radioactivity was determined using a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc., Fullerton, CA). The amount of [¹⁴C]-

malonyl-CoA formed was calculated and ACC activity was expressed as nanomoles of malonyl-CoA formed per minute per mg of supernatant protein.

FAS assay

FAS activity was measured according to a modified procedure of Smith and Abraham (1970). The sample (0.25 mg protein) was pre-incubated at 37°C for 5 minutes. The pre-incubation mixture contained 0.1 M potassium phosphate (pH 6.6), 1 mM dithiothreitol, and 0.15 mM NADPH. The reaction was started by addition of 50,000 CPM of [2-¹⁴C]-malonyl-CoA (52 Ci/mol; Moravek Biochemicals, Inc., Brea, CA) plus 55 μM of malonyl-CoA and 0.05 mM acetyl-CoA. The final reaction volume was 0.5 mL. The reaction was allowed to proceed for 20 minutes at 37°C, then terminated by addition of 0.1 mL of 30% aqueous KOH. The mixture was heated at 80°C for 30 minutes, cooled on ice and acidified with 0.3 mL of 4 N HCl. The fatty acids formed were extracted three times, each with 2 mL of hexane. The hexane extracts were combined and then evaporated at 70°C under nitrogen. After addition of 10 mL scintillation fluid, the radioactivity of the residue was measured in a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc.). The amount of fatty acids formed was calculated and FAS activity was expressed as nanomoles of palmitate formed per minute per mg of supernatant protein.

SCD assay

The Δ^9 desaturase activity was measured based on the protocol of St. John et al. (1991) with some modifications. The reaction mixture (1.0 mg microsomal protein in a total volume of 0.5 mL) contained 1.2 mM NADH, 0.1 M Tris-HCl (pH 7.4), 50,000 CPM of [1-¹⁴C]-stearoyl-CoA (55 Ci/mol; American Radiolabeled Chemicals, Inc., St. Louis, MO) plus 70 μM stearoyl-CoA, 1 mg/mL bovine serum albumin, 1.2 mM reduced glutathione, 5 mM MgCl₂, and 5 mM ATP. The solution was incubated under aerobic conditions for 20 minutes with continuous shaking at 37°C, then terminated with 0.5 mL of 10% KOH in methanol. It was then heated at 80°C for 30 minutes, cooled to room temperature and acidified with 0.5 mL of 4 N HCl. The fatty acids in the reaction mixture were extracted 3 times, each with 2 mL of hexane. The hexane was evaporated under nitrogen, and the fatty acids were methylated using 14% BF₃ in methanol. The stearate and oleate methyl esters were then separated on 10% AgNO₃-coated glass silica gel plates using hexane:diethylether (9:1) as the solvent system. The spots were visualized by spraying with water, and compared to known standards. The spot corresponding to oleate was scraped into a scintillation vial, and the radioactivity was determined in a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc.) after addition of 10 mL scintillation fluid. The SCD activity was expressed as picomoles of oleate formed per minute per mg of microsomal protein.

Statistical analysis

Data were reported as Least squares means \pm SEM. All data were analyzed using the MIXED procedure of SAS (SAS/STAT Version 6, 1998). Tukey's studentized range test was used to determine differences between treatments. Treatment

means were designated significantly different at $P < 0.05$ and were indicated by different superscripts. The following model was used for the statistical analysis:

$$Y_{ij} = \mu + D_i + E_{ij}$$

Where

Y_{ij} = observation,

μ = overall mean,

D_i = effect of diet ($i = 1, 2, 3, 4$), and

E_{ij} = residual error ($j = 6$ for LA, SA, and TVA; 5 for CLA).

RESULTS AND DISCUSSION

Body and tissue weights

Body and tissue weights of lactating dams and pups are presented in Table 3.1. Dams fed SA had lower body weights compared with those fed TVA. This was due to lower initial body weights of SA-fed dams, not due to the effect of SA supplementation, because body weight changes from d 2 to 14 were not affected among dietary treatments. Liver weights and liver weights relative to body weight of SA-fed dams were lower than those of dams fed LA, TVA, or CLA. The SA-fed dams also had lower mammary weights than those fed LA, but mammary weights relative to body weight were not affected by treatment fatty acids. Body weight of pups was not affected by dietary treatments, but weight gain (d 2 to 14) was lower in pups nursing SA-fed dams compared with LA. Liver weights of pups nursing SA-fed dams were lower than those of pups nursing TVA. Relative liver weights of pups nursing SA- and CLA-fed dams were lower than those of pups nursing TVA-fed dams.

A mixture of CLA fed to male Sprague-Dawley rats did not affect feed intake, body weight or liver weight compared with feeding linoleic acid (Sakono et al., 1999). Loo et al. (2000) fed 1% of 9/11CLA or 10/12CLA to lactating mice and observed a decrease in food intake as well as in body weight due to feeding 10/12CLA. In the present study, lactating mice were fed a restricted amount of food to avoid potential effects of the CLA isomers on food intake.

TVA and 9/11CLA content of mammary, liver, and carcass

Feeding TVA resulted in a significantly higher TVA concentration in mammary tissue, liver, and carcass of dams (Figure 3.1). Dietary TVA apparently was easily absorbed and accumulated in the tissues, but accumulation also implied that the efficiency of dietary TVA conversion to 9/11CLA was probably low.

Concentrations of 9/11CLA are shown in the lower panel of Figure 3.1. The enrichment of 9/11CLA in liver and carcass due to feeding TVA was similar to that resulting from feeding the CLA mixture. The mammary tissue accumulated 9/11CLA to a greater extent than liver or carcass, but the 9/11CLA concentration obtained from dietary TVA conversion to 9/11CLA was similar in the three tissues. Santora et al. (2000) estimated that about 11% of the TVA fed to growing mice was desaturated to CLA. They also estimated that 9/11CLA accounted for 51% of the TVA equivalents (TVA + 9/11CLA) in the tissues of TVA fed mice. Because 9/11CLA produced from TVA desaturation was found only in triglycerides, Santora et al. (2000) suggested that desaturation of TVA occurred in adipose tissue. In the present study, concentration of TVA in mammary tissue of mice fed TVA was approximately twice as much as that in liver or carcass. Similarly, 9/11CLA in mammary tissue of mice fed CLA mixture was about double that in liver or carcass. This suggested that most of TVA and 9/11CLA in mammary tissue came from the digestive tract rather than transfer from adipose tissue. This suggested that the ratio of 9/11CLA to TVA in mammary tissue of TVA-fed dams might be a good indicator of the mammary gland's capacity to desaturate TVA to 9/11CLA.

TVA and 9/11CLA content of pup carcass and liver

TVA concentration in liver and carcass of pups (Figure 3.2) indicated transfer of TVA from dams to pups via milk. Concentrations of TVA in liver or carcass of pups were similar to those in liver and carcass of the dams.

Elevated concentrations of 9/11CLA in liver and carcass of pups of CLA-fed dams (compared with LA or SA in Figure 3.2) indicated 9/11CLA was transferred via milk. Concentrations of 9/11CLA in liver and carcass (0.76 and 0.56% of total fatty acids, respectively) of pups was lower than that in mammary (1.2%), but similar to that in liver or carcass of their CLA-fed dams. Concentration of 9/11CLA in liver of pups of TVA-fed dams was intermediate between those for pups of CLA-fed dams and pups of LA- or SA- dams. This pattern is similar to the 9/11CLA concentrations observed in mammary tissue, and suggests that the 9/11CLA in tissues of pups was derived primarily from their milk supply.

Concentrations of 10/12CLA in tissues

As discussed above, feeding the CLA mixture enriched 9/11CLA content of tissues of lactating mice and their suckling pups. It is important to note that the other isomer, 10/12CLA in the mixture also was enriched in tissues (Figure 3.3). Tissues of lactating mice fed LA, SA, or TVA, and their pups, did not contain detectable amounts of 10/12CLA.

Chouinard et al. (1999b) found that abomasal infusion of 9/11CLA or 10/12CLA in lactating cows resulted in a dose-dependent transfer to milk fat. However, the transfer efficiency for the 9/11CLA was twice that for 10/12CLA. Two other studies reported similar differences in transfer efficiency (Chouinard et al., 1999a; Baumgard et al., 2000). Results of the present study further demonstrated that transfer efficiency for 9/11CLA from the diet to tissues of lactating mice also may be greater than that for 10/12CLA, because the diet provided similar amounts of each isomer.

Total fatty acid content in tissues of dams and pups

Total fatty acid (TFA) content of tissues of dams and pups in response to dietary treatments is given in Table 3.2. Compared with LA, feeding SA did not affect TFA content in mammary tissue or liver, but increased TFA in carcass. Dietary TVA did not affect mammary TFA content, but increased content of TFA in liver and carcass. Feeding the CLA mixture tended to decrease TFA content in mammary tissue, but had no effects in liver or carcass.

In pups, SA increased TFA content in liver compared with LA, but did not affect TFA in the carcass. The TVA treatment did not affect hepatic TFA content of pups, but increased TFA content in the carcass. The CLA mixture also did not affect hepatic TFA content, but decreased TFA content in the carcass.

The variable TFA response of tissues may be the result of changes in lipogenesis associated with changes in lipogenic enzyme activities. However, the restricted feeding regimen used in the present study may have caused a lower level of

lipogenesis in the tissues, especially in tissues of the suckling pups. Lower lipogenic activities may make tissues less responsive to regulation by dietary fatty acid supplementation.

Activities of ACC and FAS in mammary and liver of dams

Activities of ACC and FAS in liver of dams (Figure 3.4) were not affected by dietary treatments. In mammary tissue, however, CLA reduced ACC activity compared with other treatments. Mammary FAS activity also was not affected by dietary treatments.

As noted above, dietary fatty acids have tissue-specific effects on lipogenic enzyme activities. Abraham et al. (1983) reported that hepatic ACC and FAS activities were depressed by linoleic and linolenic acids, but not stearic or oleic acids. In contrast, ACC and FAS activities in mammary tissue were not affected by dietary polyunsaturated fatty acids. In their study, the decrease in hepatic lipogenesis paralleled decreased hepatic ACC and FAS activity. The present study indicated that the mammary tissue, but not liver, was more responsive to dietary supplementation of CLA. The indication of decreased mammary ACC activity was a 15% reduction in TFA concentration in the mammary tissue from CLA-fed dams compared with the control (Table 3.2). In their study, Abraham et al. (1983) added 4% fatty acids to a fat-free, high-carbohydrate diet, whereas the treatment fatty acids accounted for only 1% of the diet for the present study. A fat-free, high-carbohydrate diet stimulates the lipogenic pathway, whereas starvation or consumption of a diet rich in lipids decreases it (Girard et al., 1997). Restricted feeding was used in the present study and the lack of major differences in ACC and FAS activities may have been due to limited calorie intake.

Several studies demonstrated that abomasal infusion of a CLA mixture increased CLA content in milk fat (Lor and Herbein, 1998; Chouinard et al., 1999a and 1999b), but milk fat content was markedly reduced. Baumgard et al. (2000) infused pure CLA isomers and observed depressed milk fat percentage and yield (44%) when 10/12CLA, but not 9/11CLA, was infused. In the present study, the dietary CLA mixture contained 44% of 9/11CLA and 41% of 10/12CLA. Because the 10/12CLA content of mammary tissue was greater than that in liver or carcass (Figure 3.3), the 10/12CLA may have been responsible for the reduced ACC activity noted in Figure 3.4. Further research using pure isomers is needed to determine whether 9/11CLA or 10/12CLA was responsible for reducing ACC activity in the mammary gland of mice.

Activities of ACC and FAS in liver of pups

Suckling pups obtained dietary treatment fatty acids from milk of lactating dams. Dietary TVA and CLA isomers were transferred to pup tissues, but no differences in ACC or FAS activity were observed (Figure 3.5).

SCD activity in mammary and liver of dams

Hepatic SCD activity was not affected by dietary treatments (Figure 3.6). Compared with the control, dietary TVA increased SCD activity in mammary tissue.

SCD activity in liver of pups

Compared with the control, liver of pups nursing SA- or TVA-fed dams had enhanced SCD activity (Figure 3.7). Hepatic SCD activity, however, was not affected in pups of CLA-fed dams.

Results indicated that dietary TVA enriched 9/11CLA in tissues of dams. Enrichment also was observed in liver of pups nursing TVA-fed dams. Enhanced SCD activity was observed in mammary tissue due to feeding TVA, indicating the importance of SCD activity in the desaturation of TVA to 9/11CLA. Enhanced SCD activity in liver of pups from TVA-fed dams also indicated that part of 9/11CLA in liver of pups might be from endogenous conversion of TVA in milk fat.

SUMMARY AND IMPLICATIONS

Dietary TVA and CLA were absorbed and accumulated in mammary tissue, liver, and carcass of lactating dams. Dietary TVA and CLA also were transferred to liver and carcass of suckling pups via milk fat. Dietary TVA was desaturated to 9/11CLA, which was detected in mammary tissue, liver, and carcass of lactating mice. Dietary TVA caused an increase in SCD activity in mammary tissue, but not liver.

Although results of the present study did not indicate any effect of the dietary CLA mixture on SCD activity, recent studies in our and other laboratories suggested that individual CLA isomers may inhibit SCD activity. It is therefore important to investigate the effect of individual CLA isomers on SCD enzyme activity and mRNA abundance.

There is a high correlation between concentrations of 9/11CLA and TVA ($r = 0.98$) in bovine milk (Molkentin, 1999). If 9/11CLA does not depress SCD gene expression, it would not negatively affect the desaturation of exogenous TVA. Bovine milk, therefore, may be beneficial to human health by providing not only the anticarcinogenic 9/11CLA but also the substrate (TVA in milk fat) for additional 9/11CLA formation via SCD activity in human tissues.

In general, TVA and CLA caused no alterations in hepatic ACC or FAS activities in dams or their pups. Only ACC activity in mammary tissue of CLA-fed dams was reduced, and the reduction may have been due to the 10/12CLA content of the CLA mixture fed during lactation. Further research is needed to elucidate which isomer of CLA affects lipogenesis and the mechanisms of its action.

TABLE 3.1 Body and tissue weights of lactating dams and suckling pups

	DIET ¹			
	LA	SA	TVA	CLA
DAMS	6	6	6	5
Body weight ² , g	31.8 ± 0.7 ^{a,b}	30.0 ± 0.7 ^b	32.2 ± 0.7 ^a	31.8 ± 0.8 ^{a,b}
Weight change ³ , g	-6.88 ± 0.38	-7.24 ± 0.38	-7.05 ± 0.38	-8.02 ± 0.42
Liver, g	2.18 ± 0.08 ^a	1.92 ± 0.07 ^b	2.16 ± 0.07 ^a	2.17 ± 0.08 ^a
% of body weight	8.75 ± 0.20 ^{a,b}	8.45 ± 0.20 ^b	8.56 ± 0.20 ^{a,b}	9.14 ± 0.22 ^a
Mammary, g	3.46 ± 0.16 ^a	2.95 ± 0.16 ^b	3.24 ± 0.16 ^{a,b}	3.19 ± 0.17 ^{a,b}
% of body weight	13.9 ± 0.6	13.0 ± 0.6	12.9 ± 0.6	13.4 ± 0.6
PUPS				
Body weight ² , g	5.89 ± 0.40	5.63 ± 0.40	6.19 ± 0.40	6.19 ± 0.44
Weight change ³ , g	2.85 ± 0.36 ^a	1.75 ± 0.36 ^b	2.29 ± 0.36 ^{a,b}	2.42 ± 0.40 ^{a,b}
Liver, g	0.26 ± 0.04 ^{a,b}	0.18 ± 0.04 ^b	0.31 ± 0.04 ^a	0.22 ± 0.04 ^{a,b}
% of body weight	4.40 ± 0.46 ^{a,b}	3.20 ± 0.46 ^b	5.02 ± 0.46 ^a	3.31 ± 0.50 ^b

¹ LA = linoleic acid, SA = stearic acid, TVA = *trans*-vaccenic acid, CLA = conjugated linoleic acid.

² Live body weight on d 14.

³ Weight change = body weight on d 14 – body weight on d 2.

TABLE 3.2 Total fatty acid content¹ in tissues of dams and pups

	DIET ²			
	LA	SA	TVA	CLA
DAMS				
Mammary	48.7 ± 2.8 ^{a,b}	48.9 ± 2.8 ^{a,b}	52.3 ± 2.8 ^a	41.3 ± 3.0 ^b
Liver	6.52 ± 0.58 ^b	7.42 ± 0.58 ^b	9.39 ± 0.58 ^a	7.78 ± 0.63 ^b
Carcass	27.0 ± 1.2 ^b	30.3 ± 1.2 ^a	32.0 ± 1.2 ^a	25.1 ± 1.3 ^b
PUPS				
Liver	26.0 ± 10.9 ^b	56.8 ± 10.9 ^a	36.2 ± 10.9 ^{a,b}	55.1 ± 12.0 ^{a,b}
Carcass	59.9 ± 2.1 ^b	58.6 ± 2.1 ^b	67.4 ± 2.1 ^a	44.9 ± 2.1 ^c

¹mg of total fatty acids per g of tissue.

²LA = linoleic acid, SA = stearic acid, TVA = *trans*-vaccenic acid, CLA = conjugated linoleic acid.

FIGURE 3.1

Concentrations of *trans*-vaccenic acid (TVA) and *cis*9,*trans*11-18:2 (9/11CLA) in mammary tissue, liver, and carcass of lactating mice.

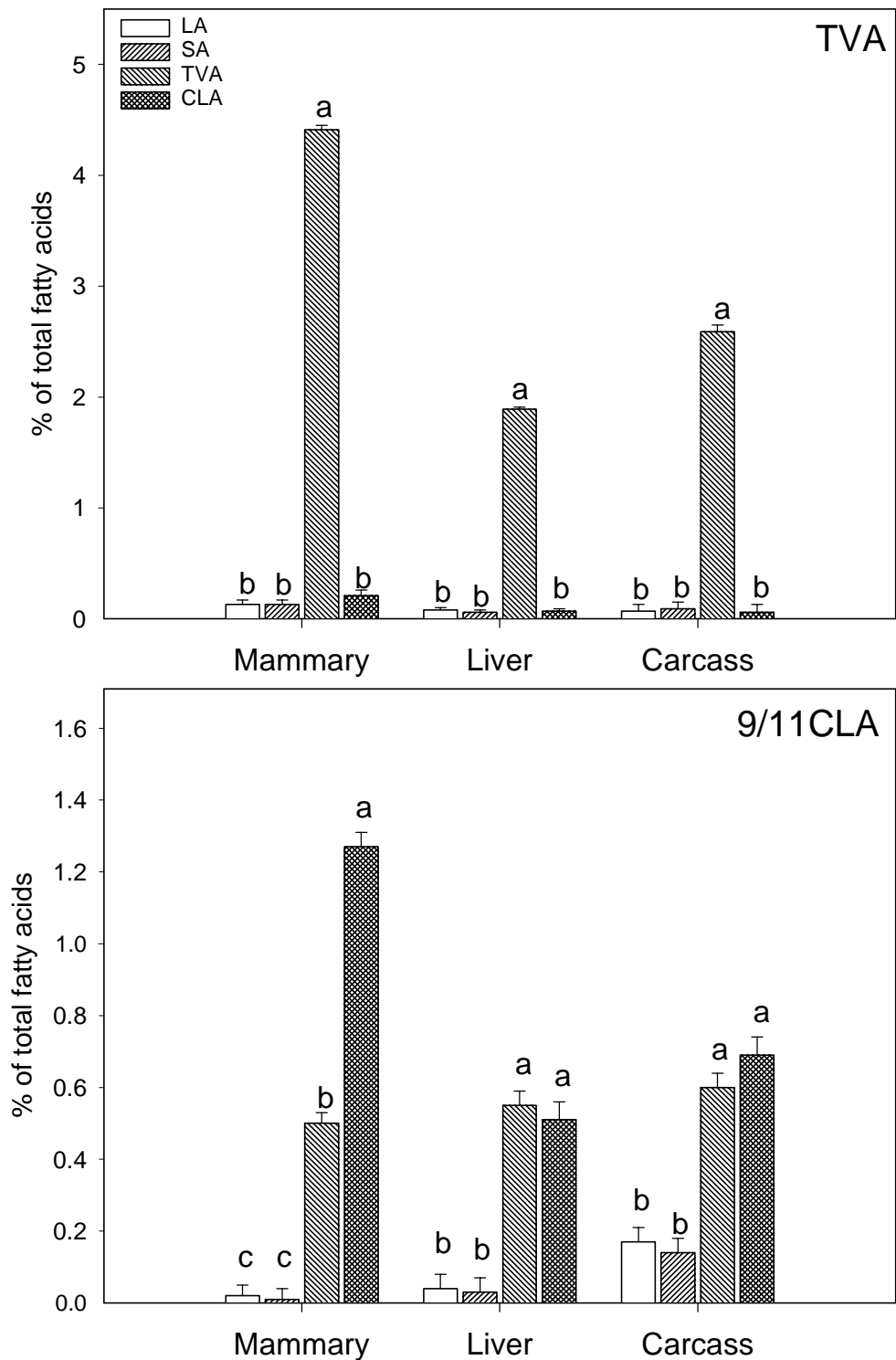


FIGURE 3.2

Concentrations of *trans*-vaccenic acid (TVA) and *cis*9,*trans*11-18:2 (9/11CLA) in liver and carcass of suckling pups.

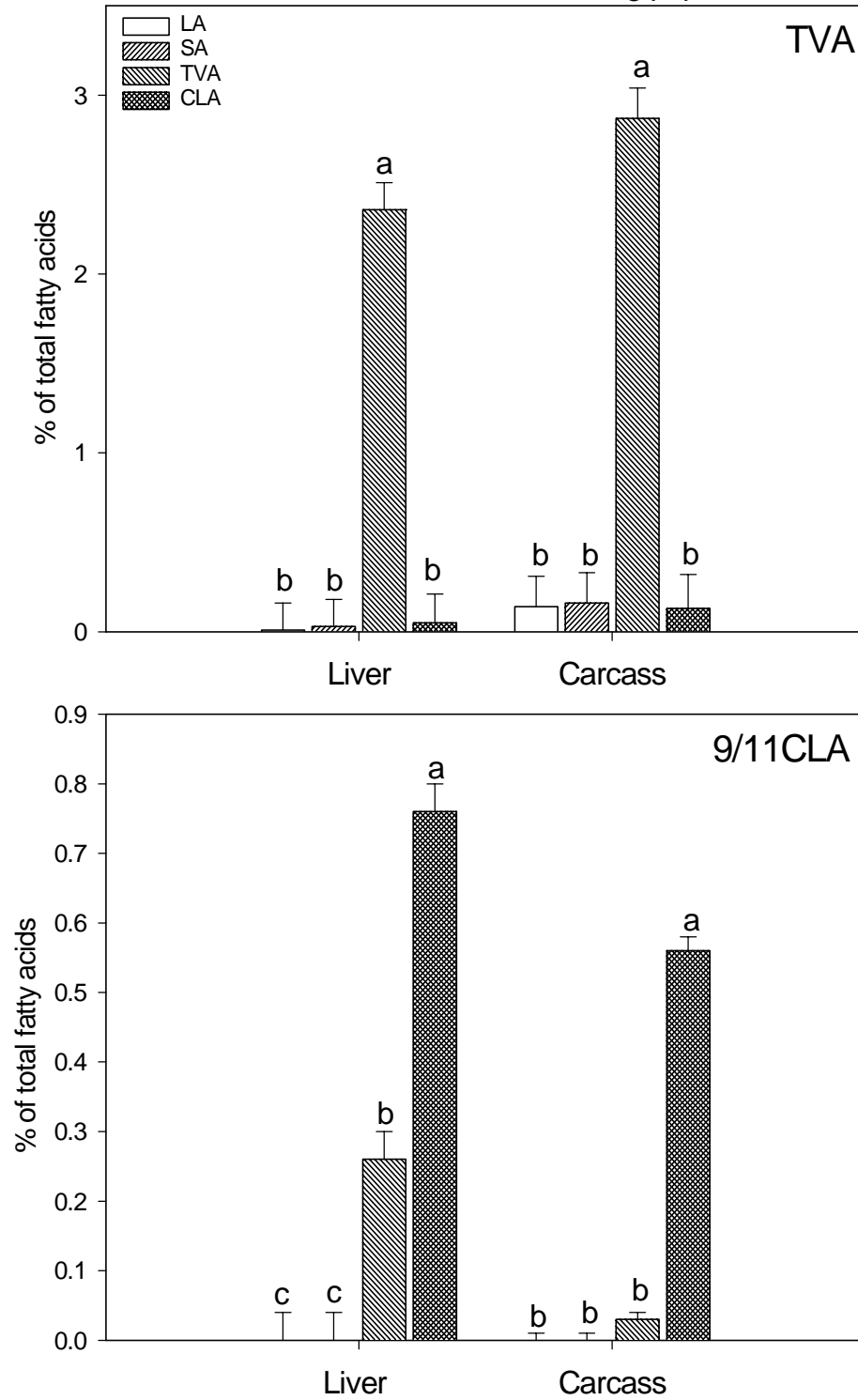


FIGURE 3.3

Concentration of CLA isomers [*cis*9,*trans*11-18:2 (9/11CLA) or *trans*10,*cis*12-18:2 (10/12CLA)] in tissues of lactating dams and their pups.

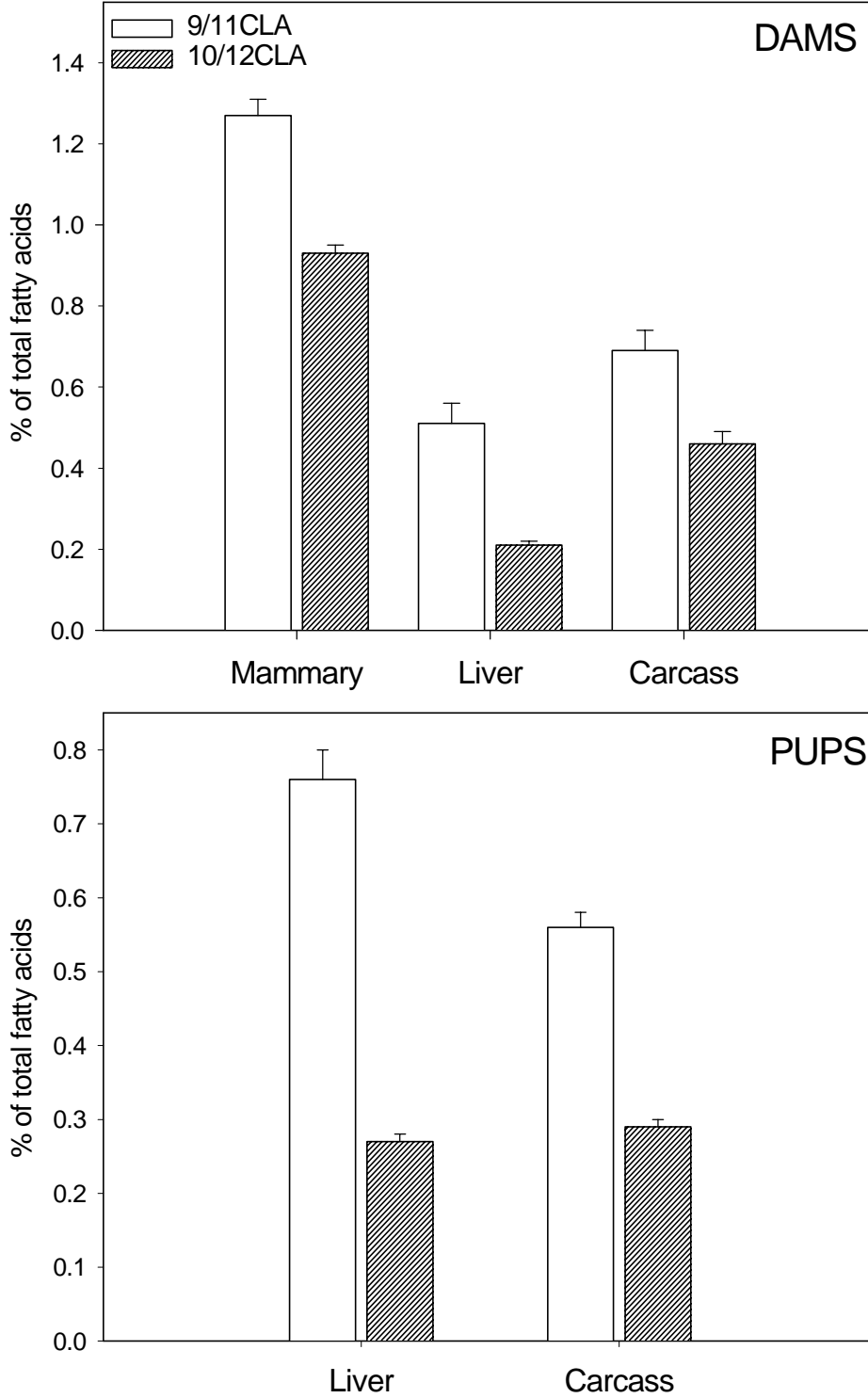


FIGURE 3.4

Enzyme activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in mammary tissue and liver.

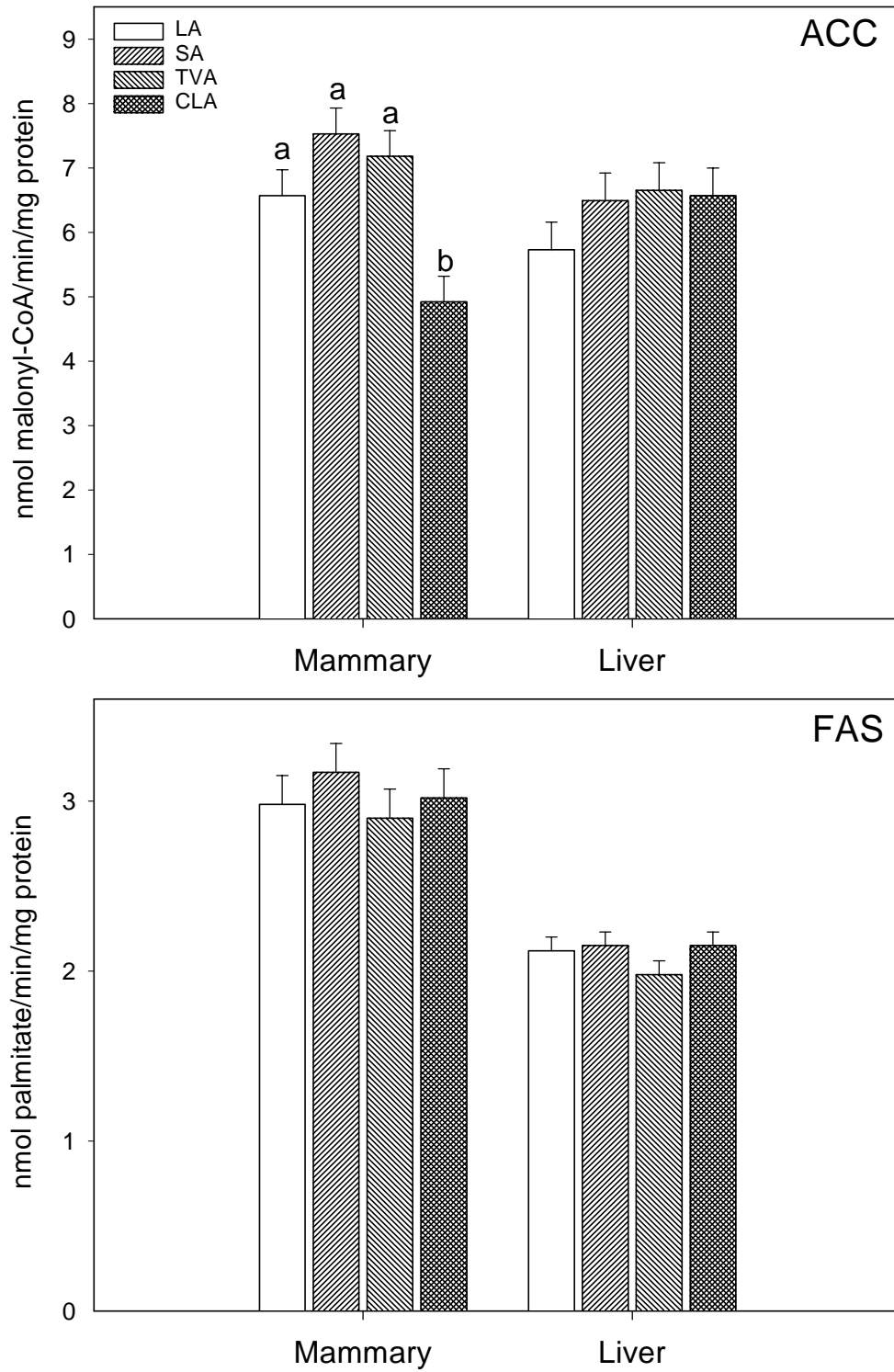


FIGURE 3.5

Enzyme activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in liver of pups.

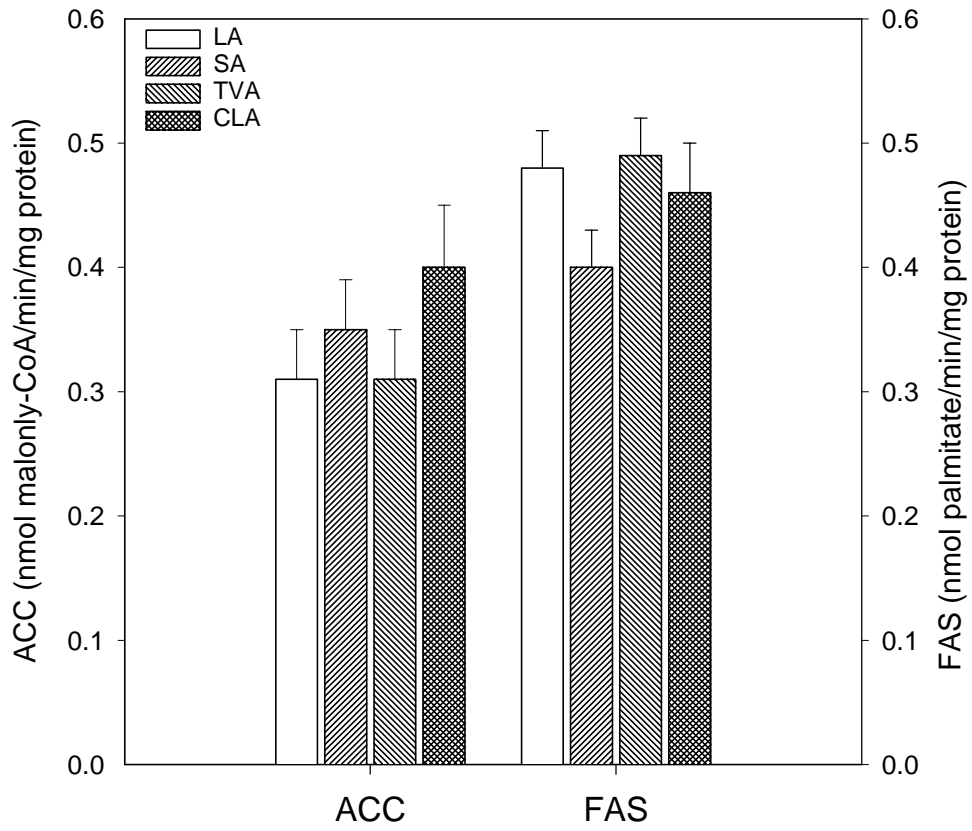


FIGURE 3.6

Enzyme activity of stearoyl-CoA desaturase (SCD) in mammary tissue and liver.

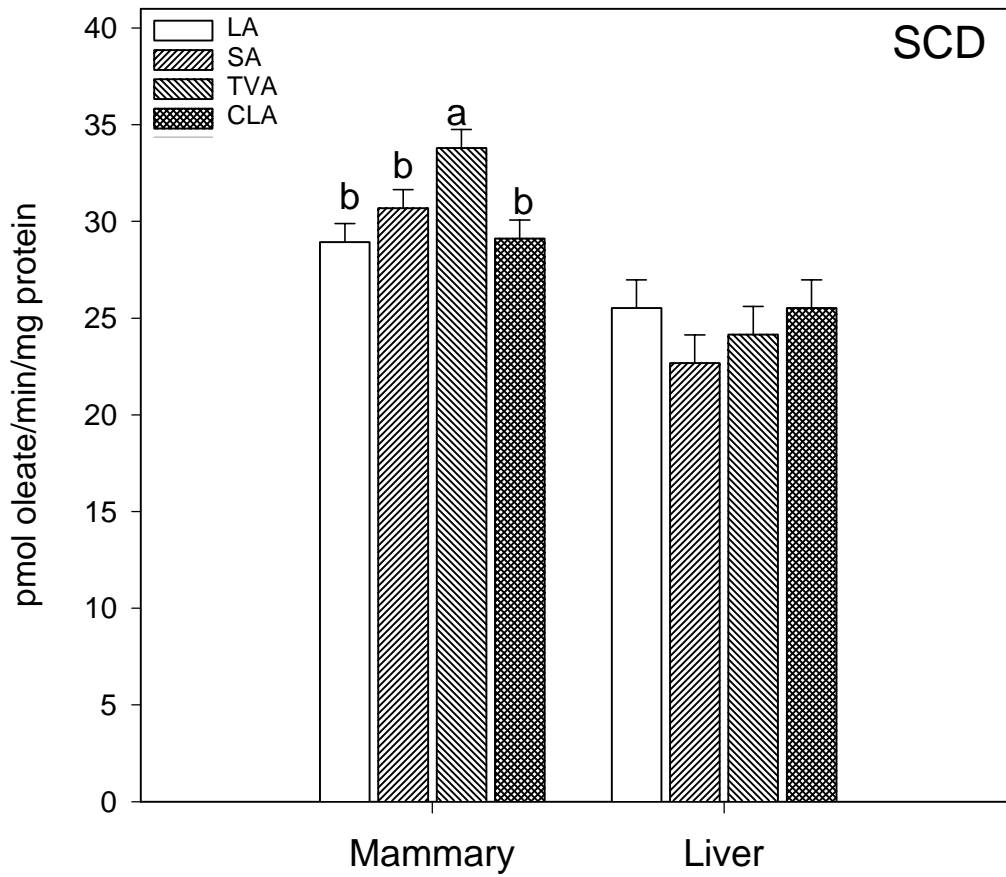
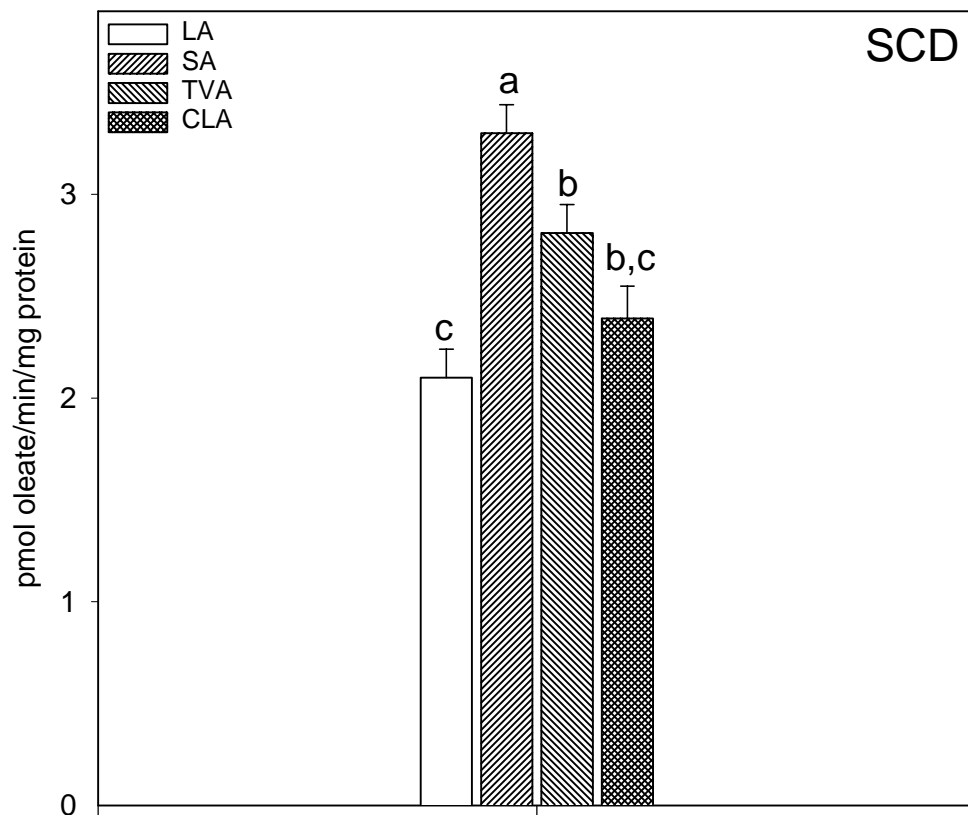


FIGURE 3.7

Enzyme activity of stearyl-CoA desaturase (SCD) in liver of pups.



CHAPTER 4

EFFECTS OF DIETARY CLA ISOMERS AND *TRANS* VACCENIC ACID ON ACTIVITIES AND mRNA ABUNDANCE FOR LIPOGENIC ENZYMES IN LACTATING MICE

ABSTRACT

To investigate the effects of two conjugated linoleic acid (CLA) isomers and *trans*11-18:1(TVA) on enzyme activities and abundance of mRNA for lipogenic enzymes in liver and mammary gland, lactating mice were fed diets containing 3% canola oil (OA) or 2% OA plus 1% stearic acid (SA), TVA, *cis*9,*trans*11-18:2 (9/11CLA), or *trans*10,*cis*12-18:2 (10/12CLA) during d 4 to 15 postpartum. Mice were sacrificed on d 15. In mammary tissue, mRNA abundance for acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD) were decreased by feeding TVA (65%, 41%, and 32% respectively), 9/11CLA (66%, 24%, and 76%), or 10/12CLA (69%, 49%, and 27%) relative to OA (100%, 100%, and 100%). Feeding SA, however, increased SCD mRNA abundance to 149% compared with OA. The two CLA isomers, but not TVA, reduced mammary ACC activity, but feeding SA increased mammary ACC activity. Mammary FAS activity, however, was not affected by dietary treatments. Reduced enzyme activity and abundance of mammary ACC mRNA due to feeding 9/11CLA or 10/12CLA were associated with lower concentrations of medium-chain fatty acids (12:0 + 14:0 + 16:0) (MCFA) in milk fat, possibly due to reduced de novo synthesis. Although CLA isomers depressed mammary ACC mRNA and enzyme activity, 10/12CLA caused greater reductions in milk fat percentage and MCFA content compared with 9/11CLA. Thus, 10/12CLA inhibited lipogenic activity of mammary tissue to a greater extent than 9/11CLA or TVA. In contrast to mammary tissue, hepatic ACC enzyme activity and mRNA abundance were not affected by dietary treatments. Although hepatic FAS mRNA was not affected by dietary treatments, TVA and 9/11CLA reduced hepatic FAS activity. Hepatic SCD mRNA also was not affected by dietary treatments, but both CLA isomers depressed hepatic SCD activity. Mammary SCD activity was reduced by TVA and CLA isomers. Pups derived treatment fatty acids from the milk of lactating dams. However, TVA and CLA isomers did not affect ACC or FAS mRNA abundance in pup liver compared with OA, as they did in mammary tissue of dams. SCD activity in the liver of pups was not affected by dietary treatments, but SCD mRNA was not detected by Northern-blot analysis. Overall, fatty acid synthesis in the mammary gland was reduced to a greater extent by 10/12CLA than by 9/11CLA; whereas desaturation capacity (SCD enzyme activity and mRNA) was reduced by TVA and both CLA isomers.

INTRODUCTION

Fatty acids regulate de novo fatty acid synthesis and desaturation in tissues such as liver. Linoleic acid depressed the rate of hepatic fatty acid synthesis in mice, and there was a 70% decrease in the activities of fatty acid synthase (FAS), malic enzyme, and glucose-6-phosphate dehydrogenase (Allmann and Gibson, 1969). More recent research also demonstrated dietary polyenoic fatty acids (n-3 and n-6) suppressed hepatic lipogenesis (Blake and Clarke, 1990). In contrast, palmitate or oleate did not affect hepatic fatty acid synthesis (Armstrong et al., 1991; Wilson et al., 1990).

Originally, Romsos et al. (1978) reported that high-fat diets fed to lactating mice for 5 days depressed lipogenesis to a greater extent in liver than in the mammary gland. In a later study, dietary linoleic and linolenic acids decreased lipogenesis and activities of acetyl-CoA carboxylase (ACC) and FAS in the liver of lactating mice, but not in the mammary gland (Abraham et al., 1983). In contrast, diets rich in polyunsaturated fatty acids reduced fatty acid synthesis in the liver as well as mammary tissue of the rat (Souza and Williamson, 1993).

Mouse mammary cell cultures provided evidence that conjugated linoleic acid (CLA) and *trans*-vaccenic acid (*trans*11-18:1) (TVA) reduced FAS activity (Jayan et al., 1998). The study also indicated CLA reduced stearoyl-CoA desaturase (SCD) mRNA abundance, whereas TVA increased both enzyme activity and mRNA abundance of SCD. In vivo, a CLA mixture reduced concentration and yield of bovine milk fat by inhibiting de novo fatty acid synthesis in the mammary gland (Lor and Herbein, 1998). Additionally, stearic acid percentage and yield in bovine milk increased in response to CLA, suggesting reduced desaturation of stearic acid via SCD in the mammary gland. Chouinard et al. (1999a) conducted a similar study and confirmed that CLA inhibited de novo synthesis and stearic acid desaturation in the mammary gland. However, the CLA mixture used in the above research contained a mixture of CLA isomers [*cis*9,*trans*11-18:2 (9/11CLA) and *trans*10,*cis*12-18:2 (10/12CLA)]. Therefore, it was not clear whether the reduction in de novo synthesis and stearic acid desaturation resulted from one or both of the CLA isomers.

The objective of this study was to evaluate the effects of purified CLA isomers (9/11CLA versus 10/12CLA) and TVA on enzyme activities and abundance of mRNA for lipogenic enzymes in liver and mammary gland of lactating mice and in liver of suckling pups. The results provided evidence for possible differences in the regulation of lipogenesis between liver and mammary gland.

MATERIALS AND METHODS

Animals

Pregnant female CD-1 mice were obtained from Harlan Teklad (Harlan, Madison, WI). Animals were maintained in the Laboratory Animal Resources facilities at Virginia Tech. Mice were fed a Harlan Teklad (Harlan) S-2335 mouse-breeder diet, which was nutritionally complete. The diet and water were provided for ad libitum consumption. Mice were housed in individual polypropylene cages in a temperature-controlled room (23°C) with a 12-h light and 12-h dark cycle. After review and approval by the Virginia Polytechnic Institute and State University Animal Care Committee, the experimental protocol was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1985).

Diets

The breeder diet (970 g) was supplemented with 30 g canola oil (OA) per kilogram diet (control) or 20 g OA plus 10 g stearic acid (SA), 10 g TVA, 10 g 9/11CLA, or 10 g 10/12CLA. The canola oil (64% oleic acid) was obtained from Hunt-Wesson, Inc. (Fullerton, CA). Stearic acid (N-18-A, 99% purity) and TVA (U-49-A, 99% purity) were obtained from Nu-Check Prep (Elysian, NY). Conjugated linoleic acid (CLA) isomers were obtained from Natural Lipids (Norway). The 9/11CLA contained 90% *cis*9,*trans*11-18:2, and 10/12CLA contained 95% *trans*10,*cis*12-18:2. Dietary groups were designated OA, SA, TVA, 9/11CLA, and 10/12CLA in tables and figures.

Design

Dams were fed the OA diet during the first 4 d of lactation. On d 4, lactating mice were randomly assigned to each dietary group, and litters were reduced to eight pups each. Only 28 of the 45 purchased mice were pregnant so the control and SA groups were assigned 5 dams each, and the other three groups were assigned 6 dams each. Unfortunately, two dams in the 10/12CLA group had to be removed from the study between d 12 and 14, because they voluntarily reduced their food intake to less than 5 g/d. Whether the sudden reduction in food intake was related to the antilipogenic effect of the 10/12CLA in the diet could not be determined.

On d 14, pups were removed from their dams 2 h before milking. Avertin (100 µL each) was injected intraperitoneally for anesthetization, followed by an intramuscular injection of 100 µL oxytocin/saline solution (1:3, vol/vol). Milk was collected by suction. On d 15, animals were anesthetized with Metofane to obtain a blood sample by heart puncture, then sacrificed by cervical dislocation. Liver and mammary glands were recovered and weighed. About 150 mg of each tissue was excised from the whole tissue, put into a labeled tube, snap frozen in liquid nitrogen, and stored at -80°C until mRNA extraction. A second portion (about 400 mg) of liver and mammary tissue was collected and stored in the same manner for enzyme assays. The remainder of the tissue was placed in liquid nitrogen and also stored at -80°C. Head, tail, skin, feet, heart, lungs, and the gastrointestinal tract were then removed. What remained constituted the carcass, which was weighed

and stored at -20°C. The carcasses were lyophilized (Dura-Top freeze dryer, FTS Systems, Inc., Stone Ridge, NY) and ground prior to fatty acid analysis. Pups within each litter were sacrificed and dissected in the manner described above. Pup carcasses within a litter were pooled for lyophilizing, grinding, and fatty acid analysis. Pup livers within a litter were also pooled for enzyme activity and mRNA analysis.

Fatty acid analysis

Milk fat contents (in percentage) were estimated by creamatocrit (Lucas et al., 1978). Milk fat was separated by centrifugation. Lipids were extracted from liver, mammary tissue, and carcass with chloroform/methanol (2:1, vol/vol). Fatty acids in tissue lipids and milk fat were directly methylated using 0.5 N NaOH in methanol, according to the base-catalyzed methylation of Park and Goins (1994). Undecenoate (Nu-Check Prep, Elysian, MN) was used as an internal standard for peak quantification. Samples were injected by auto-sampler into a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Sunnyvale, CA). Methyl esters of fatty acids were separated on a 100 m x 0.25 mm i.d. fused silica capillary column (CP-Sil 88, Chrompack, Middelburg, The Netherlands).

Preparation of samples for ACC, FAS, and SCD enzyme assays

Tissues were suspended in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, and 1 mM glutathione) at a ratio of 1 gram of tissue to 3 mL of buffer. The tissue was homogenized with a Polytron[®] homogenizer (Type PCU-2; Switzerland). All centrifugations were performed at 2°C. The homogenate was centrifuged at 5,000 x g for 15 minutes to remove nuclei, cell debris, and the fat cake on the surface. The supernatant was transferred and centrifuged for 20 minutes at 20,000 x g to pellet mitochondria. The supernatant was transferred to an ultracentrifuge tube on ice and centrifuged at 105,000 x g for 60 minutes. The supernatant thus obtained was stored at -80°C until used for assay of ACC and FAS activity. The microsomal pellet was resuspended in 0.4 mL buffer (50 mM Tris-HCl buffer, pH 7.4, and 1 mM glutathione) and stored at -80°C until analysis for SCD activity.

Microsomal protein determination

Protein contents of tissue supernatant and microsomal pellet were determined using the spectrophotometric bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL). The micro-well plate protocol as described by the reagent manufacturer was used. Absorbance was read at 560 nm on an Automated Microplate Reader (EL311s; BIO-TEK Instruments, Inc., Winooski, VT). The protein content in each sample was calculated from a standard curve plotted using known concentrations of bovine serum albumin.

ACC assay

ACC activity was assayed by the bicarbonate fixation protocol (Gregolin et al., 1966), which follows the rate of acetyl-CoA-dependent fixation of [¹⁴C]-bicarbonate

into acid-stable [^{14}C] activity (malonyl-CoA). The samples first were pre-incubated at 37°C for 15 minutes to activate the enzyme. The pre-incubation mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM sodium citrate, 10 mM MgCl_2 , 3.75 mM glutathione, and bovine serum albumin (0.75 mg/mL) in a total volume of 1 mL. The reaction was started by adding 0.4 mL of the pre-incubated sample (0.4 mg protein) to 0.4 mL of reaction mixture. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM sodium citrate, 10 mM MgCl_2 , 3.75 mM ATP, 0.125 mM acetyl-CoA, 3.75 mM glutathione, 0.75 mg/mL bovine serum albumin, 12.5 mM sodium bicarbonate, and 200,000 CPM of $\text{NaH}^{14}\text{CO}_3$ (56 Ci/mol; American Radiolabeled Chemicals, Inc., St. Louis, MO). The reaction was allowed to proceed for 10 minutes at 37°C , then terminated with 0.2 mL of 6 N HCl. Centrifugation at $15,000 \times g$ for 2 minutes was used to eliminate the insoluble material. A 0.3 mL aliquot of the supernatant was transferred to a scintillation vial, then taken to dryness at 70°C in a water bath using continuous nitrogen flow to completely remove unreacted $\text{H}_2^{14}\text{CO}_3$. After addition of 0.3 mL distilled water and 5 mL of scintillation fluid, the radioactivity was determined using a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc. Fullerton, CA). The amount of malonyl-CoA formed was calculated and ACC activity was expressed as nanomoles of malonyl-CoA formed per minute per mg of supernatant protein.

FAS assay

FAS activity was measured according to a modified procedure of Smith and Abraham (1970). The sample (0.25 mg protein) was pre-incubated at 37°C for 5 minutes. The pre-incubation mixture (0.5 mL) contained 0.1 M potassium phosphate (pH 6.6), 1 mM dithiothreitol, and 0.15 mM NADPH. The reaction was started by addition of 50,000 CPM of [$2\text{-}^{14}\text{C}$]-malonyl-CoA (48 Ci/mol; Moravsek Biochemicals, Inc., Brea, CA) plus 55 μM malonyl-CoA and 0.05 mM acetyl-CoA. The final reaction volume was 0.5 mL. The reaction was allowed to proceed for 10 minutes at 37°C , then terminated by addition of 0.1 mL of 30% aqueous KOH. The mixture was heated at 80°C for 30 minutes, cooled on ice and acidified with 0.3 mL of 4 N HCl. The fatty acids formed were extracted three times, each with 2 mL of hexane. The hexane extracts were combined and washed with 5 mL of 1 M sodium acetate (pH 5.0), then evaporated at 70°C under nitrogen. After addition of 5 mL scintillation fluid, the radioactivity of the residue was measured in a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc.). The amount of fatty acid formed was calculated and FAS activity was expressed as nanomoles of palmitate formed per minute per mg of supernatant protein.

SCD assay

Tissue Δ^9 desaturase activity was measured based on the protocol of St. John et al. (1991) with some modifications. The reaction mixture (1.0 mg microsomal protein in a total volume of 0.5 mL) contained 1.2 mM NADH, 0.1 M Tris-HCl (pH 7.4), 100,000 CPM of [$1\text{-}^{14}\text{C}$]-stearoyl-CoA (55 Ci/mol; American Radiolabeled Chemicals, Inc.) plus 70 μM stearoyl-CoA, 1 mg/mL bovine serum albumin, 1.2 mM reduced glutathione, 5 mM MgCl_2 , and 5 mM ATP. The solution was incubated under aerobic conditions for 10 minutes at 37°C , then terminated with

0.5 mL of 10% KOH in methanol. It was then heated at 80°C for 30 minutes, cooled to room temperature and acidified with 0.5 mL of 4 N HCl. The fatty acids in the reaction mixture were extracted 3 times, each with 2 mL of hexane. The hexane was then evaporated under nitrogen, and the fatty acids were methylated using 14% BF₃ in methanol. The stearate and oleate methyl esters were then separated on 10% AgNO₃-coated glass silica gel plates using hexane:diethylether (9:1) as the solvent system. The spot was visualized by spraying with water, and compared to known standards. The spot corresponding to oleate was scraped into a scintillation vial, and the radioactivity was determined in a liquid scintillation counter (LS 5000TA; Beckman Coulter, Inc.) after the addition of 5 mL scintillation fluid. The SCD activity was expressed as picomoles of oleate formed per minute per mg of microsomal protein.

Extraction of total RNA

Total RNA from liver and mammary tissue was extracted using TRI Reagent[®] from Molecular Research Center, Inc. (Cincinnati, OH), following the protocol from the manufacturer. Briefly, tissues were homogenized using a Polytron[®] homogenizer (Type PCU-2). The RNA was extracted with chloroform followed by centrifugation. The RNA was precipitated with isopropanol. After centrifugation, the RNA pellet was washed with 75% ethanol. RNA was solubilized in FORMAZOL[®] (Molecular Research Center, Inc.) and stored at -80°C. Purity of the RNA was judged by the ratio of spectrophotometric absorbance at 260 nm to that at 280 nm, and RNA was quantitated using absorbance at 260 nm. The quantity of RNA in µg/µL was obtained by the following equation:
(A₂₆₀ * Dilution factor * 40)/1000.

Alkaline downward Northern blotting

Different RNA species, including ribosomal RNA and mRNA, need to be separated. Mammary gland total RNA (30 µg) and liver total RNA (20 µg) were separated by electrophoresis through a 0.8% agarose gel containing 0.66 M formaldehyde. Since single-stranded, native RNA tends to form secondary structures, it was denatured before electrophoresis and kept in a denatured state during electrophoresis. Before electrophoresis, denaturation was achieved by heating the sample to 55°C for 15 minutes in the presence of formaldehyde and formamide. During electrophoresis of RNA, the formaldehyde added to the gel prevented formation of secondary structures (Lehrach et al., 1977). After electrophoresis, RNA was visualized by ethidium bromide staining and photographed using a Kodak Digital Science[™] DC40 Camera.

Because ACC and FAS mRNA have a size of 10.1 kb and 9.3 kb, respectively, alkaline downward capillary blotting protocol was used. The alkaline transfer buffer contained 5X SSC plus 10 mM NaOH. The 5X SSC (pH 7.4) was a 5X saturated sodium citrate, containing 0.75 M NaCl and 0.075 M sodium citrate. RNA on the gel was transferred for 3 hours to Magna Charge membrane (MSI, Westborough, MA) for mammary gland or to positively charged nylon membrane (Roche

Molecular Biochemicals, Indianapolis, IN) for liver. The RNA on the membrane was cross-linked by ultraviolet irradiation at 1200 mW/cm² for 30 seconds.

cDNA probes and Northern Blot analysis

The pKK160 plasmid, containing a 160 bp fragment of the mouse SCD1 cDNA, was a gift from Dr. James Ntambi (Department of Biochemistry, University of Wisconsin-Madison, Madison, WI). The pBR322 plasmid with an 800 bp fragment of the rat FAS cDNA was a gift from Dr. Mark Magnuson (Vanderbilt University, Nashville, TN). The plasmid pGEM-7Zf+ containing a 2.0 kb fragment corresponding to sheep ACC cDNA was kindly provided by Drs. Mike Barber and Maureen Travers (Hannah Research Institute, Scotland).

In order to obtain sufficient amounts of cDNA probes, plasmids had to be amplified, digested with appropriate restriction enzyme(s), and the cDNA probe separated. Transformation of plasmids into JM109 competent *E. coli* cells (Promega, Madison, WI) was performed using the standard heat-shock transformation protocol. Transformed cells were grown in SOC medium (0.5% (W/V) yeast extract, 2% (W/V) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) at 37°C for 1 hour. Then 50 µL or 100 µL aliquots of the culture were plated out on 100-mm diameter plates of LB agar (1% (W/V) tryptone, 0.5% (W/V) yeast extract, 1% (W/V) NaCl, and 1.5% (W/V) agar) containing appropriate antibiotics. Plates were incubated at 37°C for 18 hours. Individual colonies from the LB plates were picked, inoculated into 2 mL LB broth (the same as that of LB agar but without agar) containing the respective antibiotics for LB plates, and incubated at 37°C for 16 hours. Subsequently plasmid DNA minipreps were prepared from these broth cultures.

From the LB broth culture, 1.5 mL was pelleted by centrifugation at 4,0000 x g for 5 minutes. The cells were lysed using STET (8% sucrose, 5% triton X-100, 50 mM EDTA, and 50 mM Tris-HCl, pH 8.0) containing 0.5 mg lysozyme per mL. A miniprep of the plasmid was prepared by boiling, followed by centrifugation (12,000 x g) to pellet the precipitated chromosomal DNA and protein. Plasmid DNA in the supernatant then was precipitated using cold isopropanol at -20°C for 10 minutes, pelleted by centrifugation, washed in 70% ethanol, air dried and dissolved in 25 µL TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.4). From the miniprep, 5 µL was digested by restriction enzyme(s): *EcoR* I for ACC probe, *Pst* I for FAS, *EcoR* I and *Pst* I for SCD (double digest), and run on a 1% agarose gel to confirm the presence of the respective cDNA insert. The cDNA was visualized by ethidium bromide staining under an UV light, and identified with the help of molecular size markers. After the presence of cDNA in the vector was confirmed, the left-over transformed bacterial culture was grown in 200 mL LB broth containing the appropriate antibiotics. The crude extract of the plasmid was prepared by alkaline lysis described below.

Cells in the 200 mL LB culture were pelleted by centrifugation at 4,000 x g, then resuspended in TPG buffer (100 mM Tris-phosphate, pH 8.0, 10 mM EDTA, and

50 mM glucose) containing 2.5 mg lysozyme per mL and 100 µg/mL of RNaseA and lysed with 0.2 N NaOH containing 1% sodium dodecyl sulfate. Chromosomal DNA was precipitated with ice-cold 3 M sodium acetate (pH 5.2) and pelleted by centrifugation at 16,000 x g. Plasmid DNA in the supernatant was then precipitated with isopropanol, pelleted at 12,000 x g, washed in 75% ethanol, air dried and suspended in 3 mL TE. The plasmid was then purified by cesium chloride-ethidium bromide equilibrium centrifugation.

The crude plasmid was mixed with 3 mg ethidium bromide and 3.6 g cesium chloride, and loaded into a quick-seal ultracentrifuge tube (about 4 mL). The tube was heat-sealed and the density gradient was established by centrifugation at 90,000 rpm for 16 hours on a Beckman TLN100 rotor. After centrifugation, the plasmid band was visualized using an UV light and drawn out with an 18-gauge needle. Plasmid DNA was then precipitated with 3 M sodium acetate (pH 5.2) and 100% ethanol at -20°C overnight, pelleted by centrifugation at 12,000 x g, air-dried, and suspended in TE.

Insert DNA was excised from plasmids by restriction digestion with appropriate restriction enzyme(s). Excised inserts were separated and purified using UltraClean™15 Ultra Bind (Mo Bio Laboratories, Inc., Solana Beach, CA). Digested DNA was separated by agarose gel electrophoresis. Three volumes of potassium iodide solution were added to the gel slice containing the desired cDNA insert. Heating at 50°C for about 3 minutes melted the agarose gel slice. Ultrabind was added and the content mixed. After centrifugation at 400 x g for 1 minute, the supernatant was poured off and saved for a second purification. The pellet was resuspended in 1 mL New Wash (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 50% ethanol). The supernatant was discarded following centrifugation at 21,000 x g for 1 minute. A second centrifugation was used to completely remove any left-over New Wash. The pellet was resuspended in 10 µL TE. After a 5-minute incubation at room temperature, it was centrifuged at 21,000 x g for 1 minute. The supernatant containing the insert DNA was transferred to a new microcentrifuge tube.

Radiolabeled cDNA probes were generated using alpha-³²P[dATP] and a random priming kit (Promega) to a specific activity of approximately 1x10⁹ cpm/µg DNA. Prehybridization for both membranes was conducted for 30 minutes at 65°C (5X SSPE, 5X Denhardt's buffer, 0.1% SDS, and 100 µg/mL denatured herring sperm DNA). The 5X Denhardt's solution contained 0.1% polyvinyl pyrrolidone-360, 0.1% ficoll-400 and 0.1% bovine serum albumin. Subsequently membranes were hybridized for 16 hours with ³²P-labeled cDNA probe (5 ng/mL) specific for ACC, FAS, or SCD. High-temperature hybridization for SCD was done at 65°C (5X SSPE (pH 7.4), 5X Denhardt's, 0.5% SDS and 100 µg/mL denatured herring sperm DNA). Low-temperature hybridization for FAS and ACC was carried out at 42°C (50% formamide, 5X SSPE (pH 7.4), 5X Denhardt's, 0.2% SDS, and 100 µg/mL denatured herring sperm DNA). After hybridization with SCD probe, membranes were washed twice in 5X SSPE, 0.5% SDS at room temperature for

15 minutes each, twice in 1X SSPE, 0.5% SDS at 37°C for 15 minutes each, and once in 0.5X SSPE, 0.5% SDS at 65°C for 30 minutes (high-stringency washing). After hybridization with ACC or FAS probes, membranes were washed twice in 5X SSPE, 0.5% SDS for 15 minutes each, twice in 1X SSPE, 1.0% SDS for 15 minutes each, and once in 0.1X SSPE, 1.0% SDS for 15 minutes (medium-stringency washing).

The binding of the labeled probe to target mRNA was detected by autoradiography of the hybridized membrane on Kodak X-Omat film with two intensifying screens at -80°C. In order to ensure equivalency of loading of RNA on the gel and to quantitate mRNA bands, the membranes were stripped with a stripping solution (5 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, pH 8.0, 0.05% pyrophosphate, 0.1X Denhardt's) and re-hybridized with a labeled chicken β -actin probe. Because the membrane did not hybridize with this probe, the density of the 18S rRNA band was scanned instead. Scanning on a laser densitometer quantitated densities of bands on the autoradiograms. Density of each band was normalized to that of 18S rRNA. Relative mRNA abundance was expressed relative to that of the control group (100%).

Statistical analysis

Data were reported as Least squares means \pm SEM. All data were analyzed using the MIXED procedure of SAS (SAS/STAT Version 8, 2000). Tukey's studentized range test was used to determine differences between treatments. Treatment means were designated significantly different at $P < 0.05$ and were indicated by different superscripts. The following model was used for the statistical analysis:

$$Y_{ij} = \mu + D_i + E_{ij}$$

Where

Y_{ij} = observation,

μ = overall mean,

D_i = effect of diet ($i = 1, 2, 3, 4, 5$), and

E_{ij} = residual error ($j = 5$ for OA and SA; 6 for TVA and 9/11CLA; 4 for 10/12CLA).

RESULTS AND DISCUSSION

Body and tissue weights

The 10/12CLA isomer caused a significantly lower body weight of the dams and their pups (Table 4.1). Liver weight of the pups, but not the dams, was reduced in the group fed 10/12CLA. The 10/12CLA isomer reduced food intake compared with other treatments (data not shown), which accounted for the lower body weight of the dams. Lower body and liver weight of their pups may have been due to reduced milk production. Park et al. (1999) reported that 10/12CLA significantly reduced body weights of growing mice.

Liver and mammary weights of dams were not affected by feeding 10/12CLA, nor were their liver and mammary weights relative to body weight. Pup liver weight relative to body weight also was not affected by feeding 10/12CLA isomer.

Fatty acids in milk and tissues of dams

TVA and the two CLA isomers tended to decrease milk fat percentage, which indicated that they had the potential to reduce de novo fatty acid synthesis in the mammary gland (Table 4.2). Compared to OA, SA or TVA did not affect medium-chain fatty acid (MCFA) (12:0 + 14:0 + 16:0) concentration in milk fat. However the two CLA isomers significantly reduced MCFA concentration in milk fat. The 10/12CLA isomer decreased MCFA to a greater extent than 9/11CLA. The 10/12CLA, but not 9/11CLA, decreased MCFA in mammary tissue. Although the two CLA isomers did not alter total fatty acid (TFA) content of mammary tissue compared with the control, 10/12CLA reduced TFA content of mammary tissue compared to 9/11CLA. Because MCFA are the major products of de novo fatty acid synthesis, CLA isomers reduced MCFA concentration probably by reducing de novo synthesis. Despite the apparent inhibition of lipogenesis in mammary tissue and reduced milk fat percentage, 10/12CLA did not alter MCFA concentration in carcass of the dams but increased MCFA in their livers.

Fatty acids in tissues of pups

Dietary treatments did not affect hepatic TFA content (Table 4.2). However, compared with the control, 10/12CLA, but not 9/11CLA, reduced TFA in the carcass of pups. Compared with the control, only 10/12CLA decreased MCFA concentration in the liver and carcass of pups. Again, MCFA are mainly the end products of de novo synthesis, so a reduction in MCFA in liver and carcass may indicate reduced de novo fatty acid synthesis. However, pups from the CLA-fed dams received milk with a reduced MCFA concentration. Lower intake of MCFA in milk also may have contributed to lower MCFA in liver and carcass.

Adipose tissue also is an active lipogenic site. Compared with the control, 10/12CLA significantly reduced the MCFA concentration in the carcass of pups. A reduction in carcass MCFA concentration again may imply a reduced de novo synthesis. Dietary CLA has been shown to affect body composition changes in a number of animal species including mice, rats, and pigs (Pariza, 1999). The major sites directly affected by CLA seem to be adipocytes, the principal sites of fat

storage, and skeletal muscle cells, the principal sites of fat oxidation (Park et al., 1997). Park et al. (1999) reported that 10/12CLA, but not 9/11CLA, was associated with body composition changes (reduced body fat and increased body protein as a percentage of body weight). Observations from rat liver showed that the hypolipidemic effect of dietary CLA was in part through enhanced β -oxidation of fatty acids at the expense of esterification of fatty acids in the liver (Sakono et al., 1999).

ACC and FAS activities in mammary and liver of dams

ACC activity in liver of dams was not affected by dietary treatments (Figure 4.1). In mammary tissue, feeding SA increased ACC activity compared with the control. Feeding the two CLA isomers, but not TVA, decreased mammary ACC activity. By contrast, mammary FAS was not affected by dietary treatments. Compared with the control, feeding TVA or 9/11CLA, but not 10/12CLA, decreased hepatic FAS activity. SA did not enhance hepatic FAS activity.

ACC catalyzes the committed step in de novo synthesis of fatty acids and provides the first unique point at which control can be exerted. Although feeding SA increased ACC activity, the increase in MCFA in milk fat of SA-fed dams was not significant, nor was the increase in milk fat percentage. MCFA are the major fatty acids synthesized de novo. FAS catalyzes the conversion of acetyl-CoA and malonyl-CoA to the various end products. Feeding SA did not result in an increase in FAS activity in the mammary gland. Perhaps the rate of de novo synthesis in the mammary tissue could increase only when ACC and FAS activity increase.

Trans isomers of fatty acids are gaining more attention due to their inhibitory effect on milk fat synthesis in lactating ruminants and mice. It was suggested nearly 30 years ago that *trans* fatty acids might be responsible for the low-milk-fat syndrome of cows (Davis and Brown, 1970). It was reported that dietary as well as ruminally derived *trans* fatty acids caused milk fat depression (Wonsil et al., 1994). Baughman (1995) demonstrated that *trans*-18:1 (elaidic acid or TVA) decreased lipid accumulation in mouse mammary cell cultures. In addition, Jayan (1998) observed a decrease in cellular 16:0 in bovine mammary cell cultures associated with a decrease in ACC activity, but not FAS activity, in response to supplemental TVA. In an effort to investigate the role of *trans* fatty acids of 18:1 in milk fat depression, Griinari et al. (1998) reported that milk fat depression was associated with an increase in the *trans*10-18:1 content of milk fat, but not TVA. They postulated that the specific *trans* fatty acids affecting lipogenesis are those with a *trans*-10 double bond. Similarly, results of the present study showed that dietary TVA did not affect either ACC or FAS activity in mammary tissue in vivo. Dietary TVA did not affect MCFA concentration in milk fat or milk fat percentage.

In the initial in vivo study using lactating cows, a mixture of CLA (mainly *cis*9,*trans*11 and *trans*10,*cis*12) infused abomasally was shown to reduce bovine milk fat concentration and yield by inhibiting de novo fatty acid synthesis in the mammary gland (Lor and Herbein, 1998). A similar CLA mixture fed to lactating mice caused a decrease in mammary ACC activity (Chapter 3 of this dissertation).

Results of the present study showed that the two isomers of CLA decreased MCFA in milk fat, but the 10/12 isomer caused a greater reduction in milk fat MCFA concentration and a further decrease in milk fat percentage. This indicated that both isomers can modulate lipogenesis in the mammary tissue of lactating mice, but the 10/12 isomer appeared to be a more potent inhibitor than the 9/11 isomer. The two isomers of CLA decreased fatty acid synthesis by acting mainly on activity of ACC, but not FAS in the mammary gland. Other studies also indicated that FAS activities in mammary tissue in lactating mice or rats were not affected by dietary fatty acids (Abraham et al., 1983; Grigor et al., 1982).

ACC and FAS activities in liver of pups

ACC and FAS activities in liver of pups are shown in Figure 4.2. Pups derived treatment fatty acids from the milk of lactating mice. Feeding SA increased activity of FAS, but not ACC. Feeding TVA decreased activity of ACC, but not FAS. Compared with the control, the two isomers of CLA had no effect on activities of either ACC or FAS.

SCD Activity in mammary and liver of dams

Compared with the control, feeding SA did not alter SCD activity in mammary tissue or liver (Figure 4.3). Feeding TVA decreased SCD activity in mammary tissue, but not in liver. The two CLA isomers reduced SCD activity in mammary and liver. However, the 10/12 isomer caused a greater reduction in mammary SCD activity.

Saturated fatty acids are precursors of unsaturated fatty acids. The first step in biosynthesis of unsaturated fatty acids is the introduction of a *cis* double bond at the Δ^9 position, which is a critical step catalyzed by SCD. Therefore SCD is a key regulatory enzyme in the synthesis of unsaturated fatty acids. Although the preferred substrates are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively, SCD can also desaturate TVA to *cis*9,*trans*11-18:2 (Ntambi, 1999). Stearic acid has been associated with increased SCD activity and an increase in *cis*9-18:1 in mouse mammary cell cultures (Jayan, 1998). A physiological response to supplemental saturated fatty acids such as stearic acid would be an increase in SCD activity to maintain membrane fluidity or to facilitate triglyceride synthesis and secretion since membrane fluidity and triglyceride secretion need oleic acid. The oleic acid present in milk can be derived from the diet or desaturation of stearic acid by SCD, predominantly in liver in the case of rodents and humans (Barber et al., 1997). The lack of an effect of SA on hepatic SCD activity in the present study was most likely due to the relatively high basal amount of oleic acid in all dietary treatments.

An inhibitory effect of CLA on fatty acid desaturation was suggested by previous studies. Abomasal infusion of a mixture of CLA in Holstein cows resulted in an increase in stearic acid percentage and yield (Lor and Herbein, 1998), suggesting inhibition of stearic acid desaturation via SCD. Chouinard et al. (1999b) also reported that a mixture of CLA infused abomasally inhibited desaturation in the bovine mammary gland. Baumgard et al. (2000) infused pure CLA isomers and

attributed the inhibition of desaturation to 10/12CLA. In the present study, both isomers of CLA caused a decrease in mammary and hepatic SCD activity. However, 10/12CLA reduced mammary SCD activity to a greater extent than 9/11CLA. Therefore, 10/12CLA may be a more potent inhibitor of Δ^9 desaturation in mammary gland of lactating mice, as it was in lactating cows.

Dietary TVA reduced SCD activity in mammary tissue, but not liver. Park et al. (2000) also reported that TVA did not affect SCD activity in mouse liver. In Chapter 3, TVA increased mammary SCD activity compared to the control (3% linolenic acid), when a restricted feeding regimen was followed. Intake of dietary TVA in the present study (195 mg/d) was more than double that of the first study (75 mg/d, Chapter 3). Compared with the control (100 μ M 18:0), TVA supplemented to the medium increased SCD mRNA abundance and enzyme activity in mouse mammary epithelial cell cultures (Jayan, 1998). However, SCD enzyme activity and mRNA abundance decreased as TVA concentration increased.

SCD activity in liver of pups

Overall, there were no significant differences in SCD activity in pup liver due to dietary treatments in the dams (Figure 4.4). However, the general response in the present study was similar to that noted for pup liver SCD activity in Chapter 3 (Figure 3.7).

Abundance of mRNA for ACC and FAS in mammary and liver of dams

Abundance of ACC and FAS in the liver was not affected by dietary treatments (Figure 4.5). In mammary tissue, ACC and FAS mRNA abundance were decreased by feeding TVA (65% and 41%, respectively), 9/11CLA (66% and 24%), or 10/12CLA (69% and 49%) compared with the control (100%).

Therefore, the reduction in mammary ACC enzyme activity due to feeding the two CLA isomers could be due to lower enzyme concentration arising from a depression of mRNA levels. However, changes in FAS enzyme activity and mRNA levels did not follow the same pattern. In addition to regulation of mRNA levels by dietary factors, short-term metabolic control or even pre-translational regulation of FAS gene expression may affect FAS activity so that changes in mammary FAS enzyme activity may not follow those of FAS mRNA levels. Coupe et al. (1990) reported parallel changes in mRNA levels and enzyme activity of FAS in adipose tissue when rats were weaned to a high-carbohydrate diet. In contrast, weaning to a high-fat diet increased mRNA concentration 10- to 20-fold, but enzyme activities did not vary.

ACC and FAS mRNA in liver of pups

Abundance of ACC and FAS mRNA in liver of pups is shown in Figure 4.6. Pups derived treatment fatty acids from the milk of lactating dams, but neither TVA nor CLA isomers depressed ACC or FAS mRNA abundance in their liver when compared with the control. However, SA increased both ACC and FAS mRNA abundance. Changes in enzyme activity and mRNA levels for ACC and FAS in

liver of pups followed similar patterns. Overall, however, lesser effects from dietary fatty acids were observed in liver of pups.

SCD mRNA in liver and mammary of dams

Abundance of SCD in liver was not affected by dietary treatments (Figure 4.7). In mammary tissue, SCD mRNA abundance was decreased by feeding TVA (32%), 9/11CLA (76%), and 10/12CLA (27%) compared with the control (100%). The changes in mammary SCD enzyme activity paralleled those in mRNA levels. The reduced enzyme activities in mammary tissue could have been due to lower enzyme concentration arising from reduced mRNA levels.

Therefore, dietary fatty acids not only affected levels of SCD activity, but also modulated SCD mRNA abundance. Paisley et al. (1996) reported that nonlactating mouse hepatic SCD mRNA was decreased by dietary corn oil. Sessler et al. (1996) also observed reduced SCD mRNA in mature adipocytes by polyunsaturated fatty acids (PUFA) (arachidonic, linoleic, and linolenic acids) and it was due to a shorter half-life of the mRNA. The repression of SCD mRNA expression by PUFA has been attributed to suppression of SCD gene transcription mediated by a *cis*-acting regulatory DNA sequence in the putative promoter region of SCD genes (Waters et al., 1997). However, hepatic SCD mRNA levels were not affected by dietary fatty acids in the present study. The differences may be due to physiological state (lactation) altering gene expression in liver. Research regarding the regulation of SCD by PUFA was done either *in vitro* or by fasting and re-feeding the animals a fat-free, high-carbohydrate diet to induce high levels of SCD expression.

There is strong interest in SCD gene regulation by different fatty acids in the ruminant mammary gland. Due to biohydrogenation of dietary unsaturated fatty acids by ruminal microbes, the major fatty acid flowing to the small intestine and then the mammary gland is stearic acid. SCD activity in the mammary gland therefore plays an important role to reverse the extensive ruminal biohydrogenation of unsaturated fatty acids. As discussed previously, results of studies in which a CLA mixture or pure CLA isomers were infused indicated inhibition of desaturation in lactating cows (Lor and Herbein, 1998; Chouinard et al., 1999a; Baumgard et al., 2000). Results of the present study confirmed that 10/12CLA was the more potent of the isomers in depressing SCD mRNA in the mammary gland.

SCD mRNA in liver of pups was not detected by Northern-blot analysis. This might explain why hepatic SCD activity in pups was much lower than that in dams. Also, De Wille and Farmer (1992) reported that hepatic SCD mRNA in pups was virtually undetectable under essential fatty acids-adequate dietary conditions.

SUMMARY AND IMPLICATIONS

The preliminary study established that dietary TVA was desaturated to 9/11CLA in tissues of lactating mice (Chapter 3). Similar enrichment of 9/11CLA by feeding TVA also was observed in the present study (data not shown). The focus of the present study was to investigate the effects of TVA and the two pure CLA isomers on enzyme activity and mRNA abundance of lipogenic enzymes.

Although TVA and the two CLA isomers had the potential to reduce lipogenesis via reduced enzyme activity or mRNA in mammary tissue, only the two CLA isomers reduced MCFA in milk fat. The 10/12CLA was a more potent inhibitor of lipogenesis than 9/11CLA because it reduced MCFA concentration in mammary tissue and milk fat and caused a further reduction in milk fat percentage. The two CLA isomers reduced lipogenesis by reducing mammary mRNA abundance and enzyme activity of ACC, a critical enzyme in the lipogenic pathway. The reduction in mammary ACC activity in response to CLA also was observed in Chapter 3, therefore the reduction may have resulted from both isomers in the mixture (Figure 3.4).

Dietary TVA and the two CLA isomers also affected SCD gene expression in vivo. TVA, 9/11CLA, and 10/12CLA reduced mammary SCD enzyme activity and mRNA abundance. Because 10/12CLA caused a greater reduction than 9/11CLA, 10/12CLA may be the more potent inhibitor of desaturation in mammary gland of lactating mice.

Jayan (1998) observed that TVA stimulated SCD enzyme activity and mRNA abundance in mouse and bovine mammary cell cultures. In the same study, however, 9/11CLA decreased SCD mRNA abundance in mouse mammary cell cultures, but it increased SCD mRNA in bovine mammary cell cultures. Therefore, further research is needed to investigate whether (and if so) how CLA isomers regulate SCD gene transcription in mouse and bovine mammary epithelial cells.

TABLE 4.1 Body and tissue weights of lactating dams and suckling pups

	DIET ¹				
	OA	SA	TVA	9/11CLA	10/12CLA
DAMS	5	5	6	6	4
Body weight ² , g	45.1 ± 1.3 ^a	45.3 ± 1.3 ^a	46.8 ± 1.1 ^a	45.4 ± 1.1 ^a	41.5 ± 1.4 ^b
Liver, g	3.2 ± 0.2	3.2 ± 0.2	3.3 ± 0.2	3.6 ± 0.2	3.3 ± 0.2
% of body weight	7.1 ± 0.4	7.0 ± 0.4	6.9 ± 0.3	7.9 ± 0.3	7.9 ± 0.4
Mammary, g	5.7 ± 0.7	5.5 ± 0.7	6.1 ± 0.6	5.7 ± 0.6	6.5 ± 0.7
% of body weight	12.5 ± 1.3	12.2 ± 1.3	13.1 ± 1.2	12.5 ± 1.2	15.6 ± 1.4
PUPS					
Body weight ² , g	8.2 ± 0.4 ^a	9.0 ± 0.4 ^a	9.0 ± 0.4 ^a	8.1 ± 0.4 ^a	6.2 ± 0.5 ^b
Liver, mg	348 ± 29 ^a	359 ± 29 ^a	342 ± 26 ^a	321 ± 26 ^a	237 ± 30 ^b
% of body weight	4.2 ± 0.2	4.0 ± 0.2	3.8 ± 0.1	3.9 ± 0.1	3.8 ± 0.2

¹ OA = control, SA = stearic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2.

²Live body weight on d 15.

TABLE 4.2 Total fatty acid contents¹ and concentrations of medium-chain fatty acids (MCFA)² in tissues and milk fat percentage and MCFA concentration in milk fat

	DIET ³				
	OA	SA	TVA	9/11CLA	10/12CLA
DAMS					
Mammary					
Total	100.9 ± 9.7 ^{a,b}	102.0 ± 9.7 ^{a,b}	102.3 ± 8.9 ^{a,b}	122.3 ± 8.9 ^a	80.7 ± 10.2 ^b
MCFA	34.7 ± 1.1 ^b	36.1 ± 1.1 ^a	33.9 ± 1.0 ^{a,b}	31.6 ± 1.0 ^{b,c}	28.8 ± 1.2 ^c
Milk fat, %	28.6 ± 1.6 ^{a,b}	31.8 ± 1.6 ^a	27.2 ± 1.5 ^{b,c}	27.0 ± 1.5 ^{b,c}	24.2 ± 1.8 ^c
MCFA in milk fat	36.9 ± 1.2 ^a	38.4 ± 1.2 ^a	38.2 ± 1.2 ^a	33.2 ± 1.1 ^b	26.2 ± 1.3 ^c
Liver					
Total	22.2 ± 2.2	18.4 ± 2.2	15.6 ± 2.1	21.3 ± 2.1	24.0 ± 2.5
MCFA	26.0 ± 1.0 ^b	28.5 ± 1.0 ^a	30.3 ± 0.9 ^a	29.9 ± 0.9 ^a	29.3 ± 1.1 ^a
Carcass					
Total	119.9 ± 15.9 ^{a,b}	126.1 ± 15.9 ^a	125.3 ± 14.6 ^a	161.7 ± 14.6 ^a	79.3 ± 17.8 ^b
MCFA	27.9 ± 1.0	26.1 ± 1.0	27.4 ± 0.9	29.3 ± 0.9	26.9 ± 1.1
PUPS					
Liver					
Total	19.2 ± 1.1	18.9 ± 1.1	18.6 ± 1.0	19.0 ± 1.0	21.0 ± 1.2
MCFA	54.5 ± 0.8 ^{a,b}	56.9 ± 0.8 ^a	56.4 ± 0.8 ^a	53.4 ± 0.8 ^b	47.5 ± 0.9 ^c
Carcass					
Total	163.1 ± 9.5 ^{a,b}	182.3 ± 9.5 ^a	183.5 ± 8.7 ^a	151.0 ± 8.7 ^b	88.7 ± 10.6 ^c
MCFA	29.8 ± 0.8 ^{a,b}	32.2 ± 0.8 ^a	31.9 ± 0.7 ^a	28.6 ± 0.7 ^b	22.7 ± 0.9 ^c

¹mg of total fatty acids per g of tissue.

²MCFA = 12:0 + 14:0 + 16:0, as a percentage of total fatty acids.

³OA = control, SA = stearic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2.

FIGURE 4.1

Enzyme activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in mammary tissue and liver.

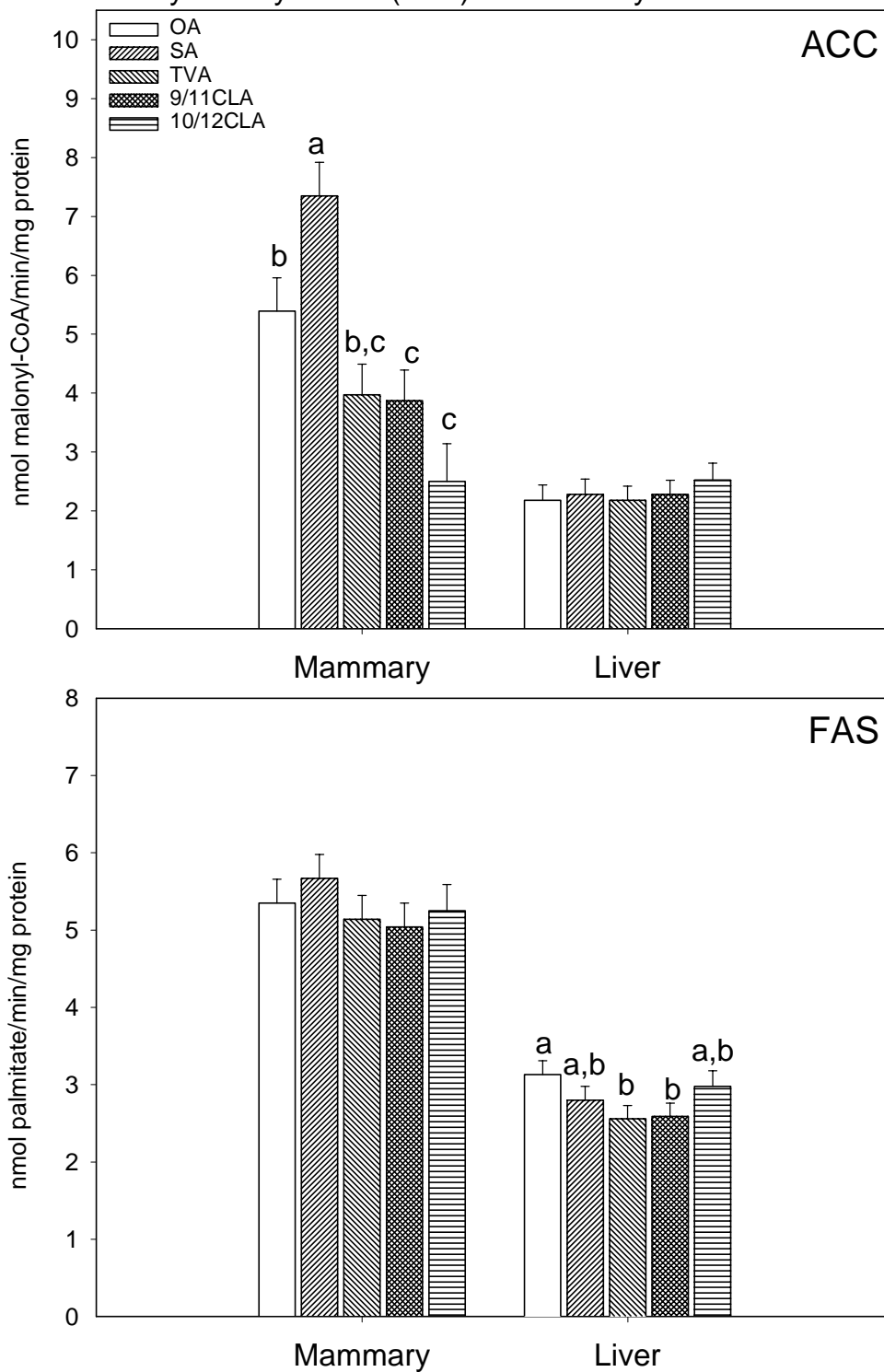


FIGURE 4.2

Enzyme activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in liver of pups.

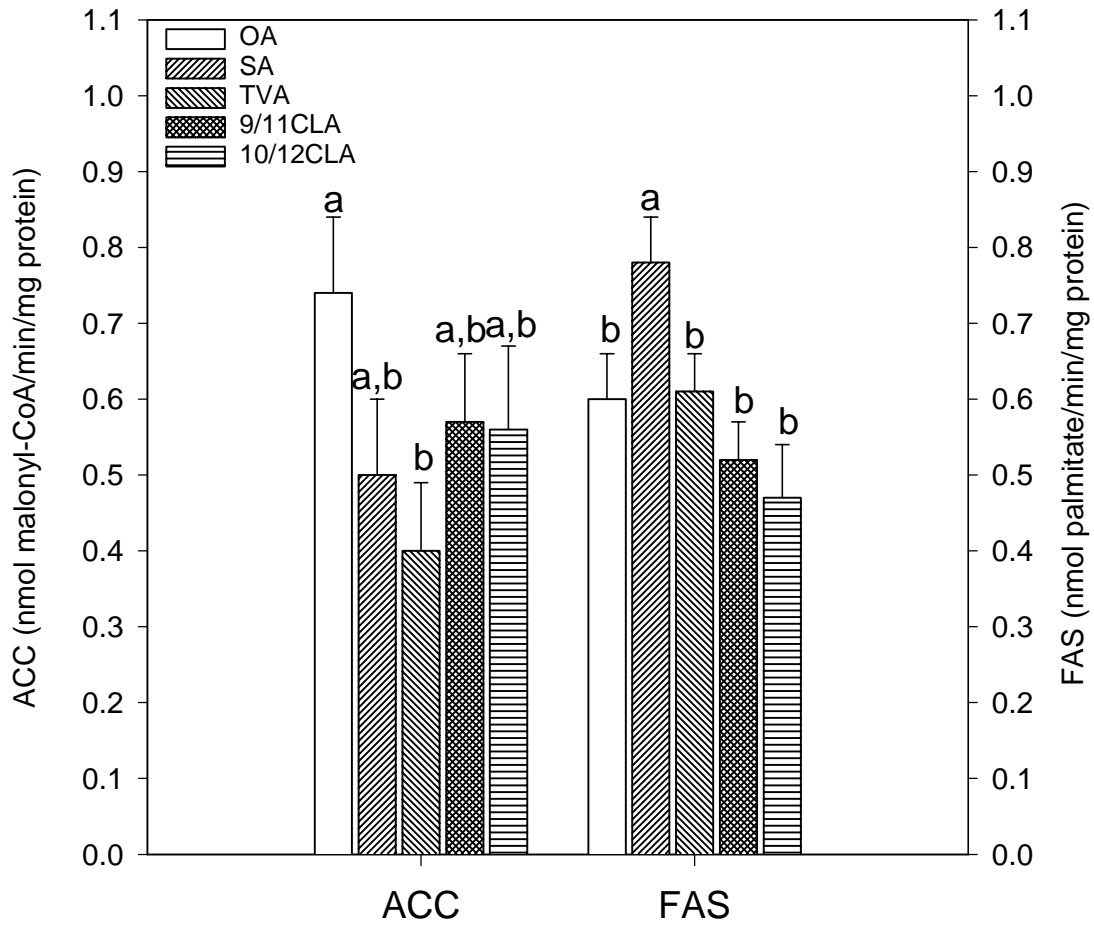


FIGURE 4.3

Enzyme activities of stearoyl-CoA desaturase (SCD) in mammary tissue and liver.

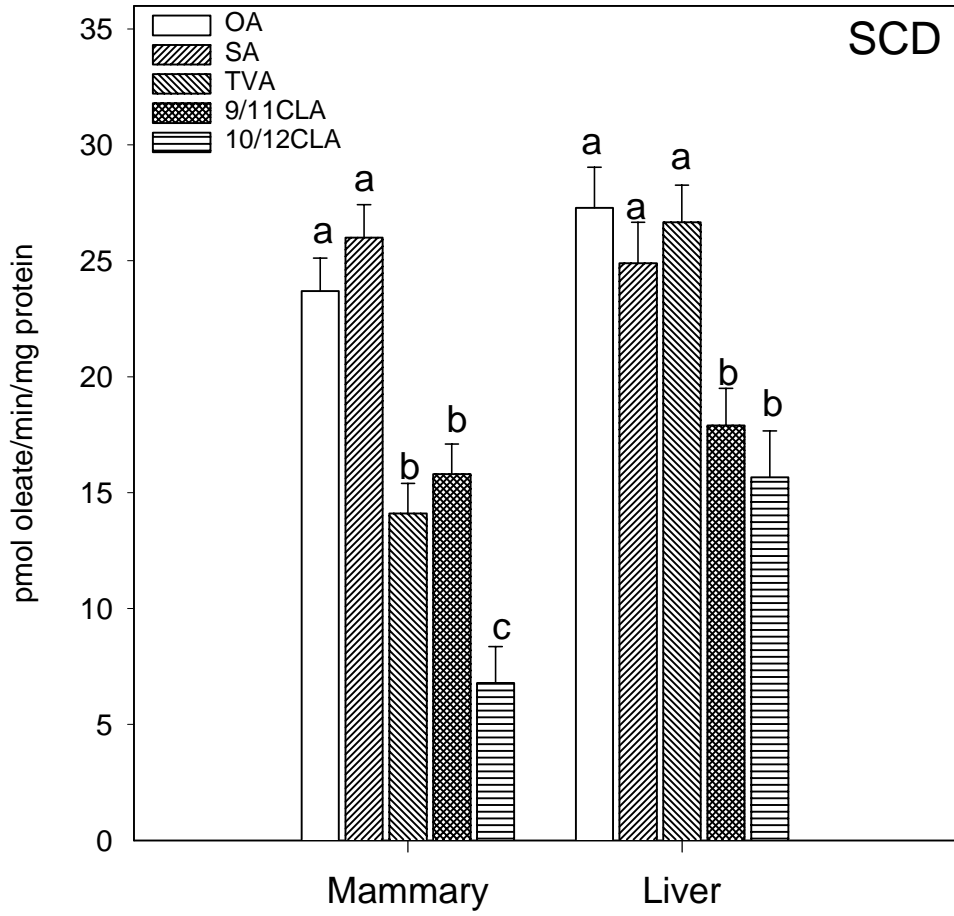


FIGURE 4.4

Enzyme activity of stearoyl-CoA desaturasae (SCD) in liver of pups.

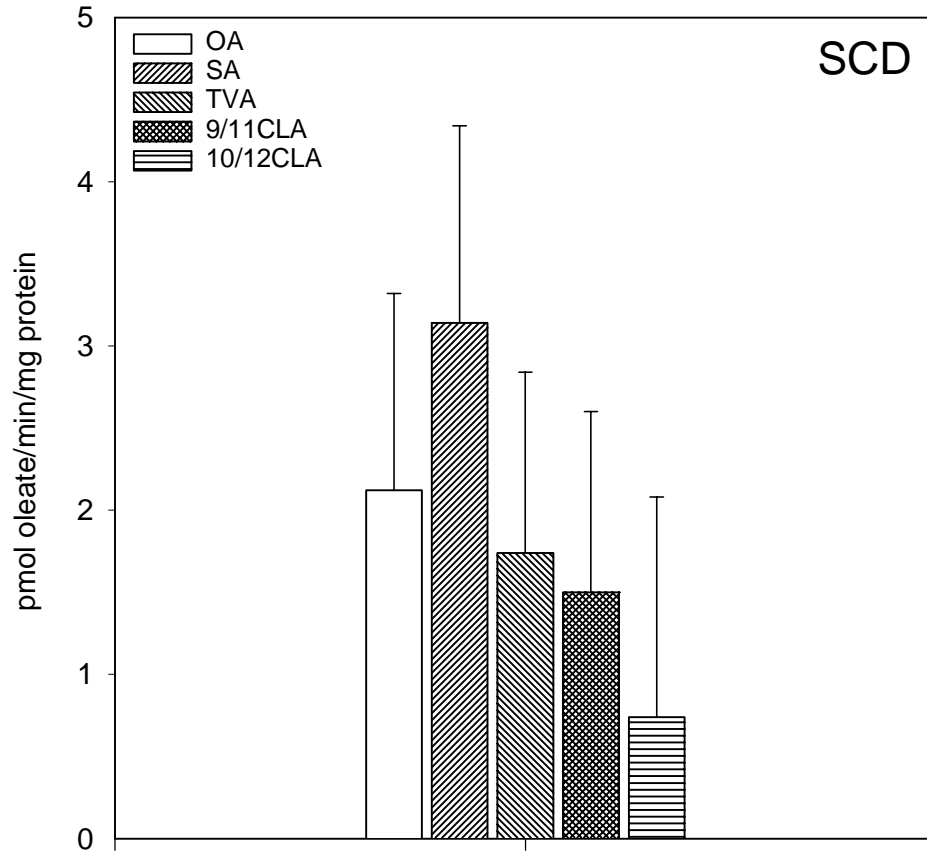


FIGURE 4.5

Abundance of mRNA for acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in mammary tissue and liver.

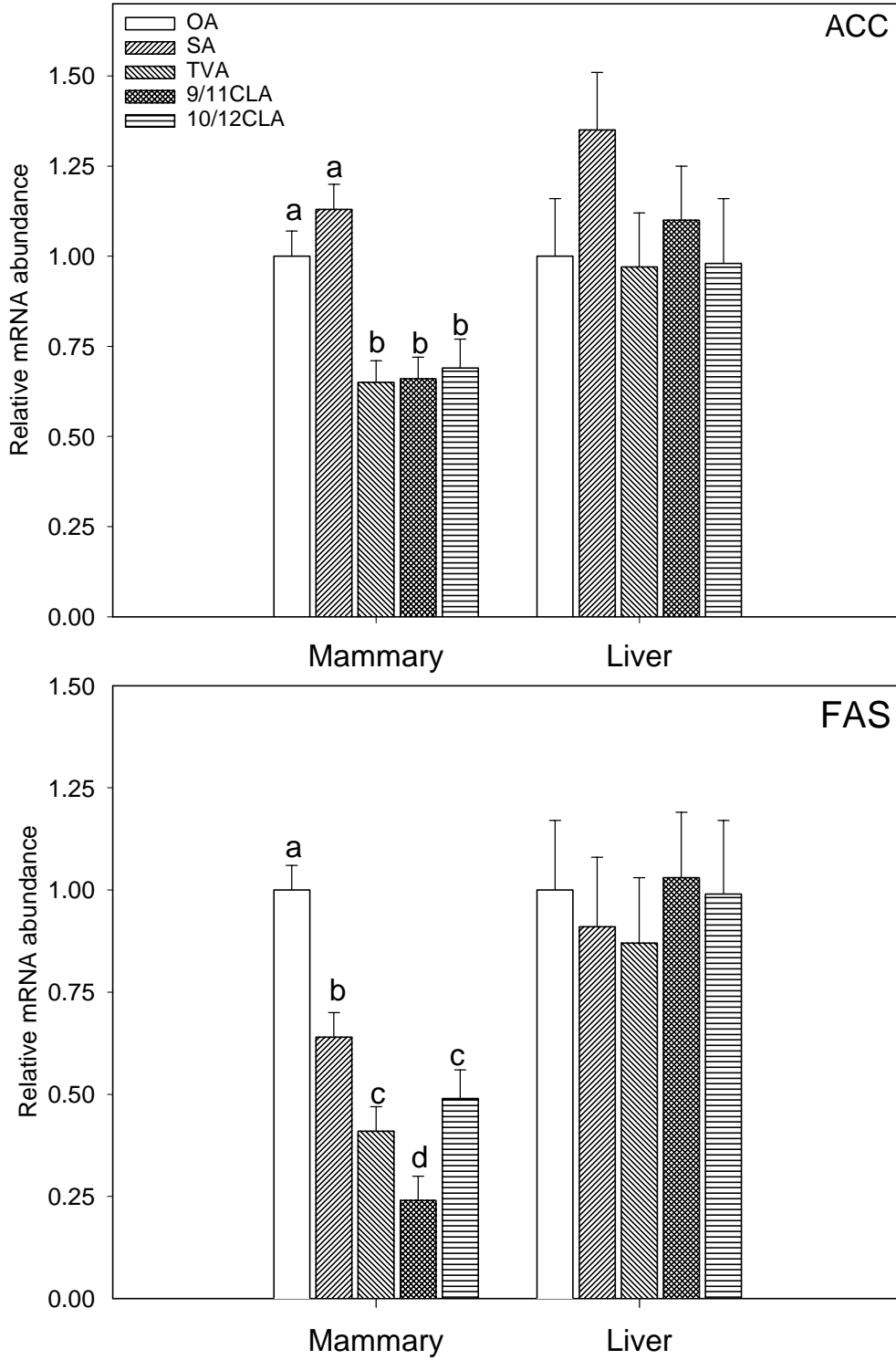


FIGURE 4.6

Abundance of mRNA for acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in liver of pups.

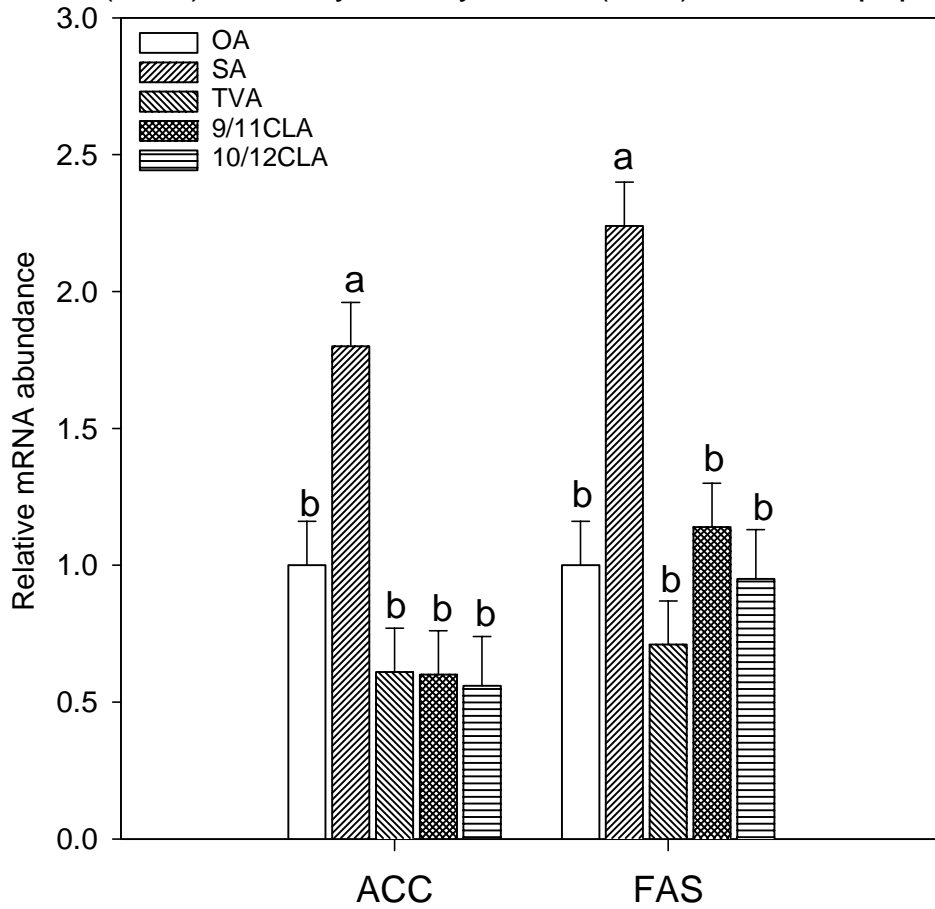
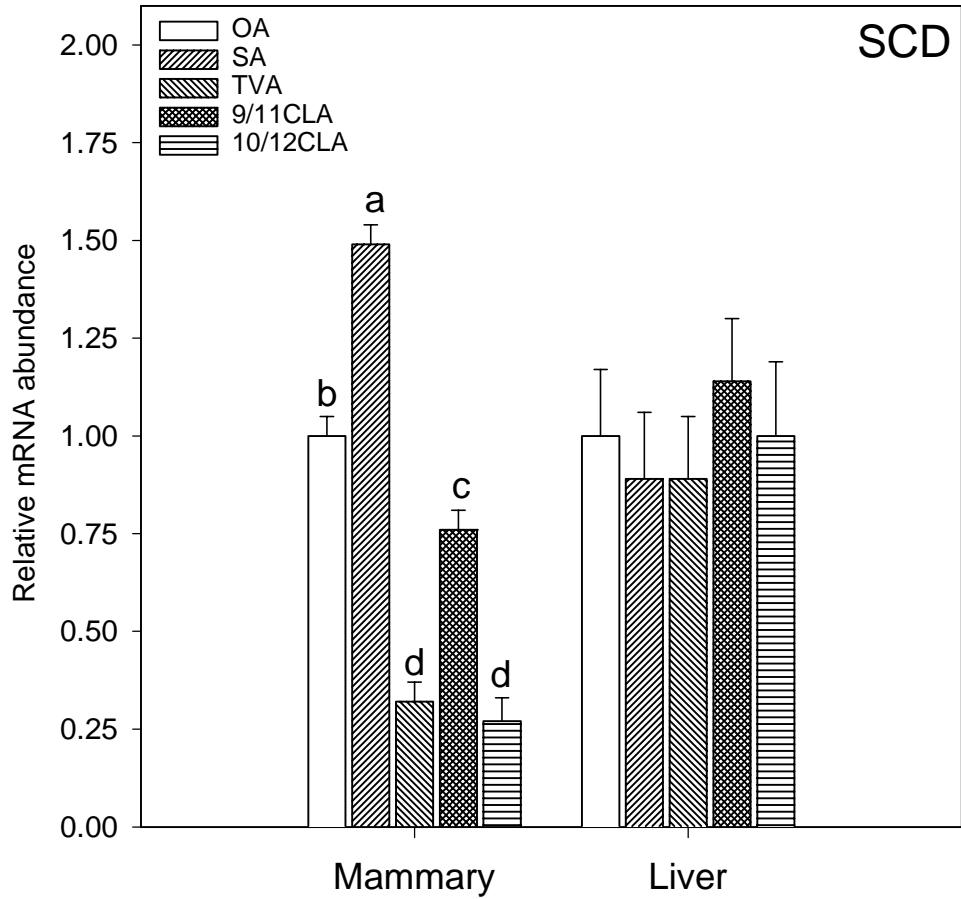


FIGURE 4.7

Abundance of stearoyl-CoA desaturase (SCD) mRNA in mammary tissue and liver.



CHAPTER 5

REGULATION OF STEAROYL-COA DESATURASE GENE TRANSCRIPTION IN MOUSE AND BOVINE MAMMARY EPITHELIAL CELLS

ABSTRACT

The objective of the study was to evaluate the effects of stearic acid, linolenic acid, and *cis* or *trans* isomers of 18:1 and 18:2 acids on stearoyl-CoA desaturase (SCD1) gene transcription in both mouse [COMMA-D/MME (MME)] and bovine (Mac-T) mammary epithelial cells. Cells were plated on 100 mm uncoated plastic Petri dishes using Dulbecco's modified Eagle's medium, harvested, and transferred to 24-well plates. Cells then were transfected with pCAT-Basic (negative control without promoter), pCAT-Promoter (positive control with SV40 promoter), or pCAT-SCD with the SCD1 promoter (600 bp). The three vectors used chloramphenicol acetyltransferase (CAT) as a reporter gene. The expression vector pCMV- β -Gal, which contained the cytomegalovirus (CMV) promoter and the gene for β -galactosidase, was cotransfected with each of the vectors as an internal control to monitor transfection efficiency. Lipofection was used to transfect mouse cells, whereas calcium phosphate co-precipitation was used to transfect bovine cells. Medium containing 0, 50, or 100 μ M of supplemental fatty acids bound to bovine serum albumin was added to the cells at the end of transfection. Supplemental fatty acid treatments included stearic acid, oleic acid, *trans*-vaccenic acid (TVA), the *cis*9,*trans*11 isomer of conjugated linoleic acids (9/11CLA), *trans*10,*cis*12-18:2 (10/12CLA), linoleic acid, or linolenic acid. The expression of pCAT-Promoter was slightly reduced only by 9/11CLA in MME, but not in Mac-T cells. The 9/11CLA isomer also reduced SCD1 gene transcription in MME cells, but not in Mac-T cells. Transcription, however, was reduced in both cell lines by 10/12CLA, linoleic, and linolenic acids. In contrast, stearic acid, oleic acid, and TVA did not affect SCD1 gene transcription. Therefore, only polyunsaturated fatty acids had the potential to depress SCD1 gene transcription and 9/11CLA only reduced SCD1 gene transcription in MME cells.

INTRODUCTION

Stearoyl-CoA desaturase (SCD) is a key enzyme involved in the biosynthesis of unsaturated fatty acids (UFA). It introduces a *cis* double bond at the Δ^9 position of long-chain fatty acids. The primary substrates for SCD are 16:0 (palmitic acid) and 18:0 (stearic acid), leading to the synthesis of *cis*9-16:1 (palmitoleic acid) and *cis*9-18:1 (oleic acid) as the two major products (Enoch et al., 1976). Jayan et al. (1998) demonstrated that oleic acid addition to mouse mammary epithelial cell culture medium resulted in a linear reduction in SCD enzyme activity and mRNA abundance; whereas stearic acid addition caused a linear increase in both enzyme activity and mRNA abundance for SCD. Another substrate for SCD is *trans*-vaccenic acid (TVA) (*trans*11-18:1), which is desaturated to *cis*9,*trans*11-18:2 (9/11CLA). When added to the mouse mammary cell culture medium, TVA stimulated SCD mRNA abundance; whereas, 9/11CLA reduced SCD mRNA abundance. Thus, substrates for the SCD reaction increased SCD mRNA, but products reduced SCD mRNA. When fed to lactating mice at 1% of intake, stearic acid increased SCD mRNA in mammary tissue as it did in vitro (Lin et al., 2000). In contrast, SCD mRNA in mammary tissue was greater in response to feeding 9/11CLA compared with feeding TVA. Therefore TVA and 9/11CLA had different effects on SCD mRNA abundance in vitro and in vivo. It is necessary to evaluate TVA and 9/11CLA on SCD at the molecular level.

Mouse hepatic SCD mRNA was repressed more than 95% by dietary polyunsaturated fatty acids (PUFA), with a corresponding 75% repression of SCD1 transcription (Ntambi, 1992). In primary rat liver cells, SCD expression was reduced by greater than 90% in response to the supplementation of 20:4n-6 or 20:5n-3 (Landschulz et al., 1994). In rat adipose tissue, SCD mRNA was repressed 75% by dietary PUFA supplementation (Jones et al., 1996). A response region to PUFA in the SCD promoter region in mouse hepatic cells has been identified (Waters et al., 1997). In their experiments, 4300, 1200, 600, 363, and 250 bp restriction fragments of the SCD 5'-flanking region were ligated upstream of the CAT (chloramphenicol acetyltransferase) gene. These constructs were then transfected into HepG2 cells and a PUFA response region between 600 bp and 363 bp was identified. This 237 bp PUFA responsive region was then used for DNA mobility shift analysis using nuclear extracts from HepG2 cells. A further 60 bp region within the 237 bp fragment was identified to have specific binding to nuclear proteins and to be necessary to mediate PUFA-specific repression.

Although studies have shown that *cis* or *trans* isomers of 18:1 and 18:2 had effects on expression of SCD (Jayan et al., 1998; Lin et al., 2000), it is not clear whether their effects on SCD enzyme activity and mRNA abundance are transcriptional or post-transcriptional. The repression of SCD by PUFA has been characterized in mouse liver, primary liver cells in culture, and rat adipose tissue, but transcriptional regulation of SCD in mammary tissue has not been evaluated. Therefore, the present study used both mouse and bovine mammary epithelial cells to investigate

the transcriptional effects of stearic acid, linolenic acid, and *cis* or *trans* isomers of 18:1 and 18:2 on SCD.

MATERIALS AND METHODS

Cell culture

Mouse mammary epithelial cells, COMMA-D/MME (MME), a subclone of the COMMA-D cell line, and bovine mammary epithelial cells (Mac-T cells), were plated on uncoated plastic Petri dishes (Corning, NY). The cells were grown to mid-log growth phase using Dulbecco's modified Eagle's medium (DMEM) (Sigma, MO). The DMEM was supplemented with 14.3 mM sodium bicarbonate and 5 mM sodium acetate and pH was adjusted to 7.2 with 1 N NaOH. The medium also was supplemented (20 mL/L) with fetal bovine serum (FBS, 2%) (Atlanta Biologicals, Atlanta), penicillin (5000 U/L) (Sigma), streptomycin (5 mg/L) (Sigma), and gentamicin (10 mg/L) (Sigma). For bovine cells, concentration of FBS was 10%. The complete medium mixture was filter-sterilized through a 0.2 μ surfactant free cellulose acetate (SFCA) filter (Nalgene, NY).

Complexing fatty acids to bovine serum albumin

Treatment media were supplemented with treatment fatty acids (FA). Stearic acid, TVA, 9/11CLA, and 10/12CLA were the same as those used in Chapter 4. Oleic acid (U-46-A), linoleic acid (U-59-A), and linolenic acid (U-62-A) were 99% pure and obtained from Nu-Check Prep (Elysian, NY). There were three concentrations (0, 50, or 100 μ M) for each fatty acid within a reporter plasmid. The supplemental FA were bound to bovine serum albumin according to Spector (1986) before they were added to the treatment medium. The molar ratio of albumin to FA was 1:2. The desired amount of FA was weighed into a 10 mL screw-capped tube and dissolved in 1 mL of hexane. Forty μ L of 1 M KOH was added to the dissolved FA to form a potassium salt of the FA. The hexane was evaporated under nitrogen by heating at 50°C for 2 minutes or until a white, chalky residue appeared. The potassium salt of the FA was dissolved in 3 mL of distilled water, and heated to 50°C in a water bath under nitrogen until the solution became clear (about 10 minutes). The warm, clear FA salt was immediately dripped with a warm Pasteur pipet over 30 to 40 seconds into 0.5 mM albumin solution in 2X PBS buffer. The pH of the solution was immediately adjusted to 7.2 with 0.2 M KOH. The solution was brought to a final volume of 50 mL with distilled water, filter-sterilized through a 0.45 μ SFCA filter (Nalgene) and stored at -80°C until use. The final concentration of BSA and FA was 0.25 mM and 0.5 mM, respectively. Just before use, the fatty acid stock solutions were diluted into DMEM to give the desired fatty acid concentrations.

Chimeric reporter constructs

All plasmid DNA were isolated and purified by cesium chloride-ethidium bromide equilibrium centrifugation (double banding) method as described in Chapter 4. The promoterless pCAT-Basic (Promega, Madison, WI) was used as the negative control. The pCAT-Promoter (Promega) containing an SV40 promoter upstream from the chloramphenicol acetyltransferase (CAT) gene was used as a positive control. The positive control was used to monitor the effects of fatty acids on transcription of the SV40 promoter. The pCAT-SCD contained the 600 bp putative

promoter region of SCD1 gene constructed upstream from the CAT gene. The pCMV- β -Gal plasmid was co-transfected with pCAT-Basic, pCAT-Promoter, or pCAT-SCD to monitor transfection efficiency.

Transfections

Because calcium phosphate-mediated transfection did not work for MME cells, they were transfected by lipofection. Calcium phosphate co-precipitation worked very well for Mac-T cells.

MME cells

MME cells were grown in 100 mm Petri dishes, then split onto Falcon[®] 24-well plates at 6×10^5 cells per well (Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to grow for 24 hours in DMEM. The medium was replaced with 0.5 mL OPTI-MEM-I (Life Technologies, Inc., Rockville, MD). In each well, cells were transfected with 0.45 μ g of total DNA (0.4 μ g of pCAT-Basic, pCAT-Promoter, or pCAT-SCD plus 0.05 μ g of pCMV- β -Gal) complexed with Lipofectamine (Life Technologies, Inc.). Lipofectamine:DNA complex may be maintained in the cell culture medium throughout the whole treatment period. However, preliminary transfection studies indicated similar transfection efficiency whether the complex was removed or not. Therefore, the Lipofectamine:DNA complex was removed after 6 hours of incubation and treatment medium containing 5% serum with treatment fatty acids conjugated to bovine serum albumin was added. There were three observations for each concentration of a fatty acid. Treatment medium was changed 24 hours following the start of transfection. Forty-eight hours following the start of transfection, cell extracts were made according to the following procedure. The cells were washed twice with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄) and incubated for 15 minutes at room temperature with 0.1 mL 1X reporter lysis buffer (Promega). Cell lysate from two wells was scraped into a microcentrifuge tube for one observation, vortexed for 10-15 seconds, then centrifuged at 21,000 x g for 2.5 minutes at 4°C. The supernatant (cell extracts) used for the CAT assay was heated at 60°C for 10 minutes to inactivate endogenous deacetylase activity. All cell extracts were stored at -80°C until analysis. The transfection process is illustrated in Figure 5.1.

Mac-T cells

Mac-T cells were grown in 100 mm Petri dishes, then split onto Falcon[®] 24-well plates at 4×10^4 cells per well (Becton Dickinson Labware) and allowed to grow for 20 hours in DMEM. The medium was changed and transfection began after an additional hour of incubation. Cells were transfected with 1.075 μ g of total DNA (1.0 μ g of pCAT-Basic, pCAT-Promoter, or pCAT-SCD plus 0.075 μ g of pCMV- β -Gal). CaCl₂ (3.2 μ L, 2M) was added to a 1.5 mL microcentrifuge tube with the plasmid DNA (1.075 μ g) in a final volume of 25 μ L. After addition of 25 μ L of 2X HEPES-buffered saline (HBS) (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na₂HPO₄) to the above mixture, the tube was vortexed for 5 seconds, then incubated for 30 seconds at room temperature. The complex (50 μ L) was dripped

to the cells. At the end of the 5-hour transfection, 15% glycerol in 1X HBS (0.2 mL each well) was given to shock the cells for 2 minutes to enhance transfection efficiency. Treatment medium (0.5 mL) with appropriate treatment fatty acids was then applied and changed 24 hours following the start of transfection. There were four observations for each level of concentration of a fatty acid. Cell extracts were made using the procedure described for MME cells. The calcium phosphate-mediated transfection process also is illustrated in Figure 5.1.

Cell extract protein determination

The protein content of cell extracts were determined using the spectrophotometric bicinchoninic acid (BCA) Protocol (Pierce, Rockford, IL). The micro-well plate protocol as described by the reagent manufacturer was used. Absorbance was read at 560 nm on an Automated Microplate Reader (EL311s; BIO-TEK Instruments, Inc., Winooski, VT). The protein content of each sample was calculated from a standard curve plotted using known concentrations of bovine serum albumin.

Beta-galactosidase enzyme assay

Beta-galactosidase enzyme activity was measured as follows. Cell extracts (25 μ L) were added to a 1.5 mL microcentrifuge tube into which 75 μ L of assay mix was added to start the reaction. The assay mix contained 0.88 mg ONPG (ortho nitrophenyl β -D-galactopyranoside), 8.75 μ moles β -mercaptoethanol, and 5.2 μ moles sodium phosphate buffer. After a 15-minute incubation at 37°C, the reaction was terminated by adding 150 μ L 1 M Na₂CO₃ and absorbance was read at 420 nm using a spectrophotometer (Model: U-2000; Hitachi Instruments, Inc., San Jose, CA). Beta-galactosidase activity was expressed as nanomoles of ONPG hydrolyzed per minute.

Chloramphenicol acetyltransferase (CAT) enzyme assay

The CAT activity was determined according to Seed and Sheen (1988) with some modifications. Briefly, cell extracts (40 μ L) were added to a 1.5-mL microcentrifuge tube with 50 μ L of assay mix added to start the reaction. The assay mix contained 0.2 μ Ci [¹⁴C]-chloramphenicol (55 Ci/mol; Moravek Biochemicals, Inc., Brea, CA) and 40 μ g of n-butyryl CoA. After a 4-hour incubation at 37°C, the reaction was terminated by adding 210 μ L of mixed xylene, vortexed for 30 seconds, and spun at 21,000 x g for 3 minutes for phase separation. The upper organic phase (xylene) was back extracted twice with 0.25 M Tris-HCl (pH 8.0). Finally, 140 μ L of the xylene phase was added to 4 mL of scintillation fluid and counted using a scintillation counter (LS 5000TA; Beckman Coulter, Inc., Fullerton, CA). CAT activity was expressed as nanomoles of chloramphenicol acetylated per minute. CAT activity was normalized to that of β -Gal and reported as nanomoles of chloramphenicol acetylated per nanomole of ONPG hydrolyzed.

Statistical analysis

Data for cellular protein, β -galactosidase and CAT activities were analyzed using the MIXED procedure of SAS (SAS/STAT Version 8, 2000). Data were reported as

Least squares means \pm SEM. SLICE option in the MIXED model was used to test the effect of different levels of a fatty acid within each reporter plasmid (pCAT-Basic, pCAT-Promoter, or pCAT-SCD). Further analysis on responses to different concentrations of a fatty acid within a reporter plasmid was performed only when the overall effect within that reporter plasmid was significant. Then, the differences were tested by non-orthogonal contrasts, and were designed as significantly different at $P < 0.05$. The contrasts were: 50 μ M versus no treatment, 100 μ M versus no treatment, and 50 μ M versus 100 μ M fatty acid treatment. The following model was used for the statistical analysis:

$$Y_{ijk} = \mu + P_i + C_j + (PC)_{ij} + E_{ijk}$$

Where

Y_{ijk} = observation,

μ = overall mean,

P^i = effect of reporter plasmid ($i = 1, 2, 3$),

C_j = effect of fatty acid concentration ($j = 1, 2, 3$),

$(PC)_{ij}$ = interaction between plasmid and fatty acid concentration, and

E_{ijk} = residual error ($k = 3$ for MME cells or 4 for Mac-T cells).

Each fatty acid was analyzed separately. There was no comparison between responses to different fatty acids. Ratios of CAT activities treated with fatty acid (50 or 100 μ M) to those untreated were calculated for pCAT-Promoter and pCAT-SCD and not analyzed statistically (Figures 5.2 and 5.3).

RESULTS AND DISCUSSION

Cell protein content

Responses to fatty acid supplementation at 0, 50, or 100 μM within a cell line were determined separately for each of the seven treatment fatty acids. Thus, statistical comparisons were only made between concentrations (0, 50, and 100 μM) within a fatty acid. Protein content of cell extracts from cells treated with fatty acids (0, 50, or 100 μM) was listed for MME and Mac-T cells in Tables 5.1 and 5.2, respectively. Protein content can be considered as an indicator of cell growth (either number or size of cells) and differences due to concentration (50 or 100 μM versus untreated) might indicate an effect on cell growth. Despite variation between different fatty acids and cell lines, there was a general trend for 9/11CLA and 10/12CLA to reduce protein content per observation. The 9/11CLA isomer only affected protein content of MME cells, but 10/12CLA reduced protein content of both cell lines.

Both cell lines are transformed cells, bearing some characteristics of tumor cells. Conjugated linoleic acids have been shown to have anticarcinogenic properties, and inhibit the proliferation of various tumor cells (MacDonald, 2000). Interestingly, 9/11CLA significantly reduced the protein content in MME cells, but not bovine Mac-T cells. Some of the physiological and molecular responses to CLA such as hepatic lipid accumulation or PPAR-responsiveness, may be species-specific (Moya-Camarena and Belury, 1999). Peroxisome proliferator-activated receptors (PPAR) have been implicated as a critical link in carcinogenesis. Fatty acids such as CLA activate PPAR and therefore regulate expression of genes important in lipid metabolism, cellular differentiation, and cancer (Vanden Heuvel, 1999). A similar response to 9/11CLA was noted in a previous study involving both cell lines (Jayan, 1998).

Beta-galactosidase enzyme activities

Activities of β -galactosidase were measured to normalize transfection efficiency of pCAT-Basic, pCAT-Promoter, and pCAT-SCD. Results in MME and Mac-T cells were listed in Appendix Tables 1 and 2, respectively. There was considerable variation in β -galactosidase activity, but there were no consistent patterns associated with cell line, plasmid, or fatty acid concentration. Based on a previous report, pCMV- β -gal is a good internal control for variation in transfection efficiency (Howcroft et al., 1997).

CAT activities

The promoterless pCAT-Basic and the pCAT-Promoter plasmids were transfected as controls for vector and cell viability changes in response to fatty acid supplementation. CAT activity was divided by β -galactosidase activity (normalized). CAT activity of pCAT-Basic ranged from 0.41 to 1.31 nanomoles of chloramphenicol acetylated per nanomole of ONPG (ortho nitrophenyl β -D-galactopyranoside) hydrolyzed in MME cells and from 0.42 to 1.14 in Mac-T cells, respectively. Because CAT activity of pCAT-Basic in response to supplemental

fatty acids was similar for all fatty acids at 50 or 100 μM , CAT activity of pCAT-Basic was subtracted from that of pCAT-Promoter and pCAT-SCD at the same concentration of a fatty acid. CAT activity of pCAT-Basic was therefore not reported. CAT activities corrected for pCAT-Basic in response to fatty acid supplementation were given in Table 5.3. Fatty acid supplementation did not affect transcription of pCAT-Promoter in Mac-T cells, but 9/11CLA caused a repression (20% at 50 μM and 24% at 100 μM , Figure 5.2) in pCAT-Promoter transcription in MME cells. This repression by 9/11CLA may result from its effect on cell growth or even the presence of a response element to 9/11CLA in the SV40 promoter.

Saturated fatty acids

From Table 5.3, it was evident that stearic acid at 50 or 100 μM did not significantly affect SCD1 transcription in either cell line. However, stearic acid enhanced SCD enzyme activity and mRNA abundance in a dose-dependent manner in MME cell cultures (Jayan et al., 1998), and stearic acid increased SCD mRNA abundance in Mac-T cells (Jayan and Herbein, 1999). Thus, in MME and Mac-T cells, stearic acid may enhance SCD mRNA abundance by increasing its stability in mammary tissue. In contrast, stearic acid did not affect SCD1 mRNA abundance in mouse liver cells (H2.35) (Ntambi et al., 1996). The investigators had previously demonstrated that dietary stearic acid did not affect hepatic SCD1 mRNA abundance in vivo (Ntambi, 1992). In addition, stearic acid did not affect SCD1 mRNA abundance in 3T3-L1 adipocytes (Sessler et al., 1996). The possibility for post-transcriptional SCD regulation by stearic acid in mammary tissue, but not hepatocytes or adipocytes, may indicate tissue-specific regulation of SCD1 by fatty acids. The observation (Lin et al., 2000) that stearic acid fed to lactating mice enhanced SCD mRNA abundance in the mammary gland, but not liver, provides further evidence for tissue-specific SCD regulation.

Monounsaturated fatty acids

Oleic acid or TVA at 50 or 100 μM did not alter SCD1 transcription in either cell line (Table 5.3). Oleic acid also did not affect SCD1 mRNA levels in mature adipocytes (Sessler et al., 1996), or in mouse liver cell culture (Ntambi et al., 1996). Oleic acid, the end product of stearic acid desaturation, may inhibit SCD activity through feedback inhibition. In mouse mammary epithelial cell cultures, oleic acid caused a dose-dependent inhibition of SCD activity (Jayan, 1998).

Trans fatty acids have received much attention due to their negative health implications. Research indicated that as dietary *trans* fatty acids increase, they accumulate in the microsomal lipids where they may impair essential fatty acid metabolism through their action on the desaturases (Mahfouz, 1981). It is very important to note that not all *trans* fatty acids affect desaturases, the position of the double bond in the *trans*-18:1 is important in determining the degree of inhibition (Mahfouz et al., 1980a). The impaired desaturation and chain elongation of the essential fatty acids was related particularly to the consumption of *trans*9-18:1 (elaidic acid) (Molkentin, 1999).

Interestingly, TVA enhanced enzyme activity and mRNA abundance for SCD in MME and Mac-T cell cultures, and the concentration of the product of TVA desaturated by SCD (9/11CLA) was increased (Jayan et al., 1998; Jayan and Herbein, 1999). An in vivo study (Loor et al., 1999) demonstrated that dietary TVA enriched 9/11CLA in blood plasma, liver, mammary tissue, and carcass of lactating mice when their food intake was restricted to 9 g/d. The dietary TVA also increased SCD activity in the mammary gland (Loor et al., 1999). In a second study (Loor et al., 2000; Lin et al., 2000), dietary TVA again increased 9/11CLA in liver, carcass, and milk fat of lactating mice when their food intake was not restricted. In this study, however, dietary TVA did not enhance SCD enzyme activity or mRNA abundance.

The primary *trans*-18:1 isomer in bovine milk, TVA, can be desaturated via SCD to 9/11CLA, an anticarcinogen. With regard to human health implications, the fact that TVA did not depress SCD transcription is very important. If human mammary tissue is similar to mouse or bovine, then the rate at which lactating women convert TVA to 9/11CLA via SCD will not be affected by TVA content of the diet. Thus, greater intake of TVA by lactating women should result in greater amounts of the anticarcinogenic 9/11CLA being synthesized for transport to their child via milk.

Polyunsaturated fatty acids

*Trans*10,*cis*12-18:2 (10/12CLA), linoleic acid, and linolenic acid at 50 and 100 μ M depressed SCD1 transcription in both cell lines (Table 5.3). At 50 μ M, they repressed SCD1 transcription in MME cells by 82%, 75%, and 80%, respectively (Figure 5.2). At 100 μ M, the repression was 91%, 90%, and 85%. At 50 μ M, the three fatty acids depressed SCD1 transcription in Mac-T cells by 74%, 70%, and 76% (Figure 5.3). At 100 μ M, the depression was 85%, 86%, and 81%. Although repression of SCD1 transcription was numerically greater at 100 μ M, the differences were not significant. By contrast, 9/11CLA caused a significant reduction in SCD1 transcription in MME cells at both concentrations (56% and 67%, respectively), but not in Mac-T cells. However, 9/11CLA also caused a depression in the transcription of pCAT-Promoter plasmid in mouse cells.

A great deal of research has demonstrated that hepatic SCD1 is transcriptionally repressed by PUFA. An in vitro study with primary cultured hepatocytes showed that linoleate, arachidonate (20:4), and eicosapentaenoate (20:5) depressed SCD1 transcription (Landschulz et al., 1994). An in vivo study demonstrated that SCD1 transcription was suppressed by PUFA, but not saturated or monounsaturated fatty acids (Ntambi, 1992). However, the PUFA response is not liver specific. Jones et al. (1996) reported that feeding a high-PUFA (48% corn oil) diet significantly decreased SCD1 mRNA in adipose tissue. In their in vitro study, the authors showed a dose-dependent downregulation of SCD1 mRNA by PUFA in 3T3-L1 adipocytes. Sessler et al. (1996) also observed that linoleic, linolenic, eicosapentaenoic, and arachidonic acids decreased SCD1 mRNA stability in a dose-dependent manner (80% maximum repression).

It is clear that most research on regulation of SCD1 by PUFA has been focused on liver and adipose tissue. Kinsella (1970) did not detect desaturase activity in the mammary glands of lactating rats and rabbits. It therefore had been postulated that the mammary gland of monogastric animals receive an adequate supply of UFA from blood plasma to facilitate formation of a milk fat globule that is liquid at physiological temperatures. Calabro et al. (1982) confirmed that SCD activity in microsomes from lactating rat mammary glands was very low regardless of stage of lactation, indicating that the oleic acid present in milk of lactating rats was derived from desaturation of stearic acid by SCD in the liver. However, lactating mice showed a level of desaturase activity in the mammary gland that was equal to that found in the livers of non-lactating mice (Rao and Abraham, 1974). SCD activity in the mammary tissue of lactating ruminants is important, because the mammary gland receives primarily stearic acid resulting from biohydrogenation of UFA in the rumen. Without desaturation of stearic acid in the bovine mammary gland, semisolid milk fat globules would form at body temperatures (Kinsella, 1970). Bovine liver apparently has little SCD activity (St. John et al., 1991). Although SCD activity in ruminant adipose tissue is high, it was reported that the SCD gene was repressed in adipose tissue during pregnancy and lactation in sheep (Ward et al., 1998). Results of the present study indicated that an elevated supply of PUFA would reduce SCD gene transcription in bovine and mouse mammary tissue.

Linoleic and linolenic acids depressed the transcription of SCD1 in mouse and bovine mammary epithelial cells. Jayan et al. (1998) observed that linoleic acid inhibited enzyme activity and mRNA abundance for SCD at 50 and 100 μM in MME cells. In mammals, stearic acid is a precursor of oleic acid, which in turn is a precursor of n-6 PUFA. SCD catalyzes the introduction of the first *cis* double bond at the Δ^9 position, a critical committed step in the synthesis of n-6 UFA. SCD activity affects the balance and/or the ratio between saturated and unsaturated fatty acids in cellular membrane phospholipids. This ratio directly influences the membrane fluidity and its physical properties. Therefore, linoleic and linolenic acids may reduce SCD activity by depressing its transcription to allow regulation of membrane fluidity in mammary cells.

Although there have been some studies on the effects of CLA on SCD enzyme activity and mRNA levels, there are no reports on the effects of CLA on SCD1 gene transcription. A mixture of CLA (primarily 9/11CLA and 10/12CLA) infused abomasally demonstrated that CLA inhibited *de novo* synthesis as well as desaturation in the mammary gland (Chouinard et al., 1999b). Lee et al. (1998) reported that a dietary supplement containing a similar mixture of CLA isomers decreased rat hepatic expression of SCD mRNA. Their *in vitro* study in H2.35 liver cells showed that the same mixture, but not the pure 9/11CLA, significantly suppressed SCD1 mRNA, implying that 10/12CLA was responsible for the depression of SCD1 mRNA both *in vivo* and *in vitro*. Direct inhibition of mouse liver SCD activity was caused by 10/12CLA, but not 9/11CLA (Park et al., 2000). Pure 9/11CLA did not affect hepatic SCD mRNA but depressed SCD1 mRNA in

mammary tissue in lactating mice (Lin et al, 2000). This indicated that 9/11CLA may affect SCD1 mRNA expression in the mammary tissue, which at least partly explained a low conversion of dietary TVA to 9/11CLA in the mouse mammary tissue (Loor et al., 2000). In contrast to its depression of SCD1 gene transcription in mouse mammary cells, 9/11CLA did not repress SCD1 transcription in bovine Mac-T cells. This suggested that ruminants might have evolved to cope with substantial quantities of 9/11CLA from the rumen. Then increasing the output of both TVA and 9/11CLA from the rumen can increase 9/11CLA in bovine milk fat, because TVA can be desaturated to 9/11CLA. The 9/11CLA isomer, either from the rumen or from desaturation of TVA, will not affect the action of SCD in mammary gland of lactating dairy cows.

In lactating mice, dietary 10/12CLA depressed SCD1 mRNA abundance to a greater extent than 9/11CLA (Lin et al., 2000). This study indicates that the results may have been due to the greater inhibition of SCD1 gene transcription by 10/12CLA compared with 9/11CLA. However, 10/12CLA did not affect SCD1 mRNA levels in liver of lactating mice, implying that mammary tissue in lactating mice is more sensitive to fatty acid regulation. The effects of two relatively pure CLA isomers (9/11 and 10/12) on milk fat synthesis and desaturation were investigated in lactating dairy cows (Baumgard et al., 2000). They found that 10/12CLA was the primary isomer inhibiting de novo synthesis as well as desaturation in the mammary gland. In agreement with their observations, 9/11CLA did not depress SCD transcription in bovine Mac-T cells.

SUMMARY AND IMPLICATIONS

Stearic acid did not affect SCD transcription in either cell line. The observed increase in SCD mRNA abundance may be due to increased mRNA stability. Oleic or TVA also did not affect SCD transcription. Similar to stearic acid, they may affect SCD mRNA or enzyme activity by post-transcriptional regulation. Linoleic acid, linolenic acid, and 10/12CLA significantly depressed SCD transcription in MME and Mac-T cells. The 9/11CLA, however, did not affect SCD transcription in Mac-T cells, which suggests TVA desaturation to 9/11CLA is not regulated by 9/11CLA concentration in the bovine mammary gland. Although 9/11CLA reduced pCAT-Promoter transcription, the depression of pCAT-SCD plasmid in MME cells suggested that 9/11CLA might depress SCD gene transcription in mouse mammary cell cultures.

Besides depressing Δ^9 desaturation, 9/11CLA and 10/12CLA also may decrease Δ^5 or Δ^6 desaturation of linoleic acid. Linoleic acid is desaturated and elongated to arachidonic acid, the major precursor of eicosanoids. Eicosanoids are involved in cancer, immune function, and cardiovascular diseases. CLA have been reported to have beneficial effects on these diseases and CLA may exert their effects by affecting eicosanoid metabolism. An in vitro study (Bretillon et al., 1999) with rat hepatic microsomes demonstrated that 9/11CLA was more active than 10/12CLA in reducing Δ^6 desaturation of linoleic acid. This observation is consistent with the consensus that 9/11CLA is the biologically active isomer, especially in terms of its anticarcinogenic properties. However, 10/12CLA appeared to be more potent than 9/11CLA in reducing Δ^6 desaturation when infused into the abomasum of lactating dairy cows (Lor and Herbein, 2000). Therefore, 9/11CLA and 10/12CLA may also demonstrate species differences in reducing Δ^6 desaturation.

Overall, results of the present study indicated that *cis* or *trans* isomers of 18:1 may not depress SCD gene transcription. Repression of SCD gene expression by PUFA is not limited to liver or adipose tissue. Mammary SCD gene transcription is also affected by PUFA. Results of the present study indicated that the 600 bp promoter region of SCD gene may contain response elements to PUFA in mammary epithelial cells. Further research using a longer SCD promoter may reveal more information on its transcriptional regulation by fatty acids.

TABLE 5.1 Protein content ($\mu\text{g}/\text{observation}$) of cell extracts in response to fatty acid supplementation in MME cells transfected with reporter plasmids

Fatty acid ¹	pCAT-Basic	pCAT-Promoter	pCAT-SCD	SEM ²
SA-0	113.36	118.93	117.14	
SA-50	105.01	109.22	106.75	4.92
SA-100	106.38	112.78	109.56	
OA-0	97.58	97.16	95.47	
OA-50	94.77	92.62	96.65	4.68
OA-100	95.86	87.64	104.96	
TVA-0	89.77 ^{a3}	82.33	83.29	
TVA-50	70.90 ^b	73.82	69.21	5.12
TVA-100	88.54 ^a	82.00	70.66	
9/11CLA-0	80.40 ^a	80.42 ^a	73.38 ^a	
9/11CLA-50	56.84 ^b	55.67 ^b	53.00 ^b	5.30
9/11CLA-100	75.14 ^a	58.25 ^b	62.53 ^{a,b}	
10/12CLA-0	93.46 ^a	83.21 ^a	80.41 ^a	
10/12CLA-50	44.35 ^b	48.04 ^b	44.74 ^b	4.38
10/12CLA-100	58.56 ^c	48.21 ^b	50.56 ^b	
LA-0	97.58	97.16	96.87	
LA-50	98.97	96.82	96.65	3.76
LA-100	95.86	87.64	106.19	
LAN-0	115.77	89.16	96.94	
LAN-50	105.35	84.69	85.94	4.24
LAN-100	117.45	96.80	97.26	

¹ SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

² Pooled SEM.

³ Different superscripts (a, b, c) indicate significant differences at $P < 0.05$ between responses to concentrations within a fatty acid of a reporter plasmid.

TABLE 5.2 Protein content ($\mu\text{g}/\text{observation}$) of cell extracts in response to fatty acid supplementation in Mac-T cells transfected with reporter plasmids

Fatty acid ¹	pCAT-Basic	pCAT-Promoter	pCAT-SCD	SEM ²
SA-0	102.89	93.65	73.94	
SA-50	70.04	70.44	53.37	11.90
SA-100	70.36	76.97	47.07	
OA-0	77.12	83.60	58.76	
OA-50	69.05	77.96	52.33	6.87
OA-100	70.80	74.15	48.39	
TVA-0	59.72	63.85	51.64	
TVA-50	60.67	74.15	47.34	4.69
TVA-100	63.27	70.67	53.57	
9/11CLA-0	59.49	45.14	29.58	
9/11CLA-50	53.66	40.35	22.74	5.29
9/11CLA-100	52.58	48.56	17.34	
10/12CLA-0	85.90 ^{a3}	68.31 ^a	57.65 ^a	
10/12CLA-50	61.66 ^b	54.16 ^b	45.67 ^b	3.75
10/12CLA-100	65.46 ^b	53.40 ^b	52.26 ^{a,b}	
LA-0	71.96	58.20	47.75	
LA-50	67.92	65.45	37.16	7.90
LA-100	68.34	79.24	36.00	
LAN-0	59.48	56.44	72.44	
LAN-50	48.26	43.45	58.64	7.30
LAN-100	45.71	46.18	54.93	

¹ SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA is linoleic acid, and LAN = linolenic acid.

² Pooled SEM.

³ Different superscripts (a, b) indicate significant differences at $P < 0.05$ due to concentration of a fatty acid within of a plasmid.

TABLE 5.3 Activities¹ of CAT in response to fatty acid supplementation in MME and Mac-T cells

Fatty acid ²	pCAT-Promoter		pCAT-SCD	
	MME	Mac-T	MME	Mac-T
SA-0	19.60 ± 0.44	16.74 ± 0.46	3.54 ± 0.44	2.93 ± 0.46
SA-50	19.43 ± 0.44	16.50 ± 0.46	3.36 ± 0.44	2.78 ± 0.46
SA-100	18.94 ± 0.44	17.18 ± 0.46	3.39 ± 0.44	2.91 ± 0.46
OA-0	27.53 ± 1.36	13.67 ± 0.33	3.26 ± 1.36	2.61 ± 0.33
OA-50	26.00 ± 1.36	14.28 ± 0.33	3.36 ± 1.36	2.27 ± 0.33
OA-100	27.62 ± 1.36	14.07 ± 0.33	3.02 ± 1.36	2.20 ± 0.33
TVA-0	20.74 ± 0.62	14.09 ± 0.35	2.69 ± 0.62	2.52 ± 0.35
TVA-50	19.96 ± 0.62	13.88 ± 0.35	3.01 ± 0.62	1.98 ± 0.35
TVA-100	20.62 ± 0.62	13.67 ± 0.35	2.82 ± 0.62	2.15 ± 0.35
9/11CLA-0	17.79 ± 0.50 ^{a3}	12.69 ± 0.43	3.77 ± 0.50 ^a	3.20 ± 0.43
9/11CLA-50	14.22 ± 0.50 ^b	12.27 ± 0.43	1.65 ± 0.50 ^b	2.95 ± 0.43
9/11CLA-100	13.47 ± 0.50 ^b	11.55 ± 0.43	1.26 ± 0.50 ^b	2.41 ± 0.43
10/12CLA-0	21.45 ± 1.15	12.53 ± 0.33	4.34 ± 1.15 ^a	3.66 ± 0.33 ^a
10/12CLA-50	20.91 ± 1.15	12.25 ± 0.33	0.80 ± 1.15 ^b	0.95 ± 0.33 ^b
10/12CLA-100	19.02 ± 1.15	12.13 ± 0.33	0.41 ± 1.15 ^b	0.53 ± 0.33 ^b
LA-0	24.21 ± 0.81	11.26 ± 0.46	4.25 ± 0.81 ^a	3.08 ± 0.46 ^a
LA-50	24.16 ± 0.81	11.72 ± 0.46	1.06 ± 0.81 ^b	0.91 ± 0.46 ^b
LA-100	22.99 ± 0.81	11.65 ± 0.46	0.42 ± 0.81 ^b	0.43 ± 0.46 ^b
LAN-0	18.96 ± 1.49	14.85 ± 0.50	5.96 ± 1.49 ^a	3.69 ± 0.50 ^a
LAN-50	17.89 ± 1.49	14.76 ± 0.50	1.19 ± 1.49 ^b	0.87 ± 0.50 ^b
LAN-100	17.90 ± 1.49	14.99 ± 0.50	0.92 ± 1.49 ^b	0.71 ± 0.50 ^b

¹ Nanomoles of chloramphenicol acetylated per nanomole of ONPG (ortho nitrophenyl β-D-galactopyranoside) hydrolyzed.

² SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

³ Different superscripts (a, b) indicate significant differences at $P < 0.05$ due to concentration of a fatty acid within a plasmid.

FIGURE 5.1

Transfection of MME cells by lipofection or Mac-T cells using calcium phosphate mediated protocol.

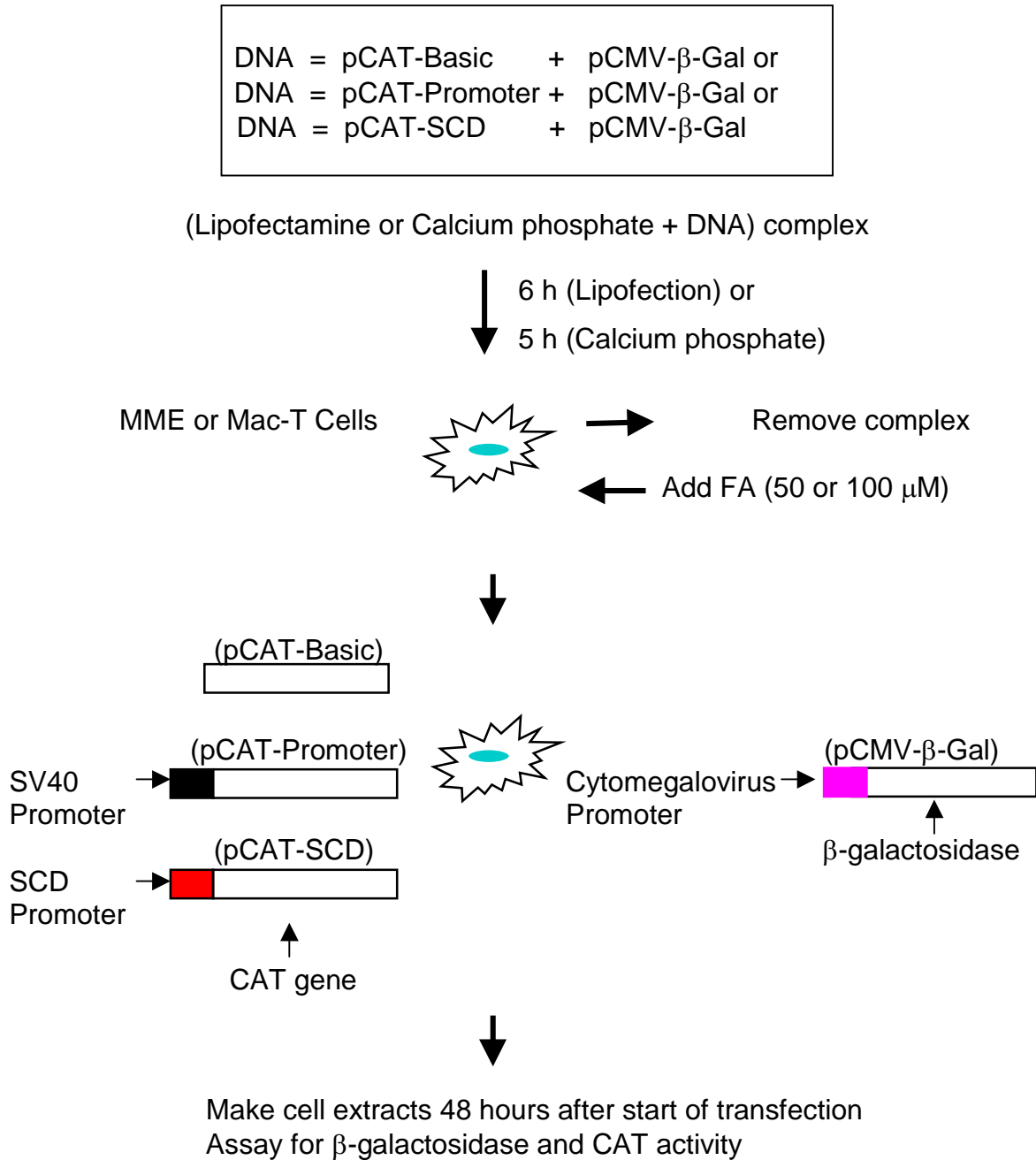
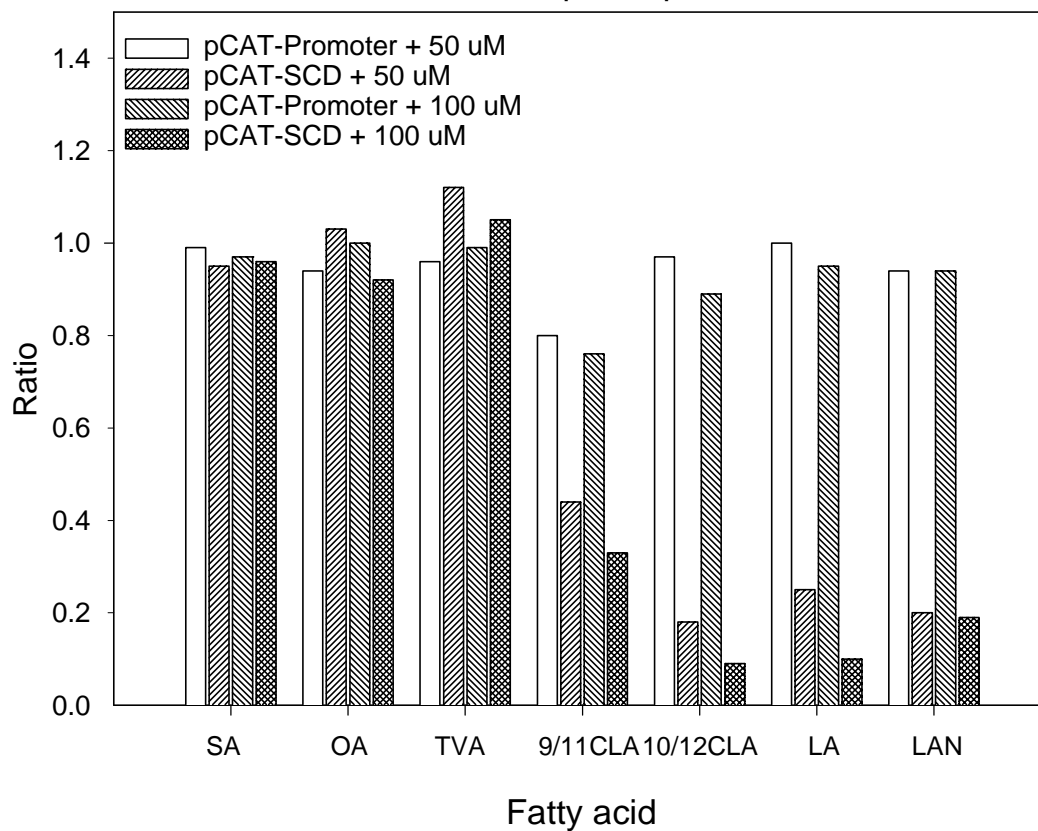


FIGURE 5.2

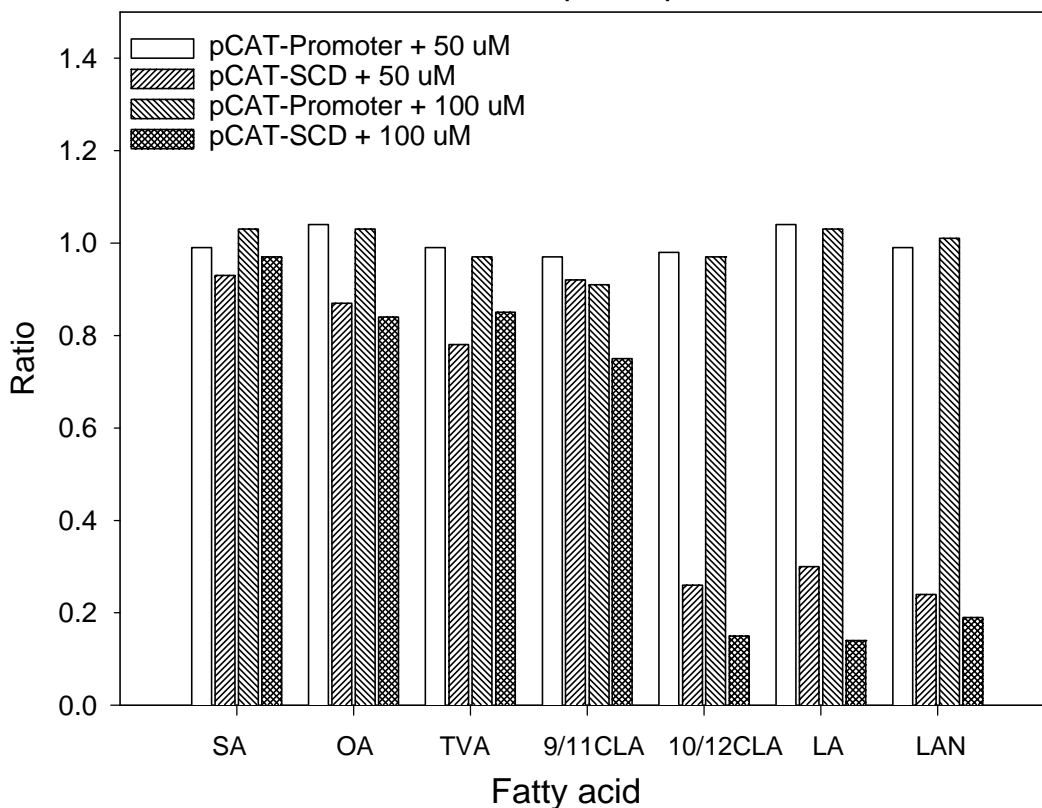
Ratio of CAT activity at 50 or 100 uM of a fatty acid to that at 0 uM within the same reporter plasmid in MME cells.



SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

FIGURE 5.3

Ratio of CAT activity at 50 or 100 uM of a fatty acid to that at 0 uM within the same reporter plasmid in Mac-T cells.



SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

CHAPTER 6

OVERALL CONCLUSION AND IMPLICATIONS

Bovine milk fat contains more than 70% saturated fatty acids, the majority of which result from de novo synthesis in the mammary gland. Bovine milk fat is somewhat unique, compared to non-ruminant species, because it contains substantial quantities of *trans*-vaccenic acid (*trans*11-18:1) (TVA) and the anticarcinogenic isomer of conjugated linoleic acid (*cis*9,*trans*11-18:2) (9/11CLA). The mammary gland and other tissues have the capacity to desaturate TVA to form 9/11CLA via stearoyl-CoA desaturase (SCD). Typically, there is a positive correlation between TVA and 9/11CLA concentrations in bovine milk fat, indicating desaturation of TVA may be a primary source of the 9/11CLA found in bovine milk and dairy products.

Results of the first experiment indicated that dietary TVA was desaturated to 9/11CLA in lactating mice. Enrichment of 9/11CLA occurred in mammary gland, liver, and carcass of lactating dams. A similar enhancement in 9/11CLA concentration was observed in liver of suckling pups nursing TVA-fed dams, indicating transfer of 9/11CLA from dams to pups via milk. An increase in SCD activity in response to dietary TVA supplementation was observed in mammary tissue. Thus, diets supplemented with TVA not only provide substrate for synthesis of anticarcinogenic CLA; they also may stimulate activity of the enzyme for anticarcinogenic CLA synthesis.

Results of the second experiment indicated that the 9/11CLA and *trans*10,*cis*12-18:2 (10/12CLA) reduced de novo fatty acid synthesis by reducing mammary ACC enzyme activity and mRNA abundance. However, 10/12CLA caused greater reductions in medium-chain fatty acid concentration and milk fat percentage than 9/11CLA. Results of the two experiments and recent reports from other laboratories indicate 10/12CLA is a potent inhibitor of body fat deposition and milk fat synthesis. Thus, 10/12CLA may someday be used to regulate the ratio of lean to fat in animals grown for meat production. Results of the second experiment, however, suggest it should not be supplemented in the diet of lactating animals.

A slight increase in mammary SCD activity due to dietary TVA was observed in the first study. In contrast, dietary TVA decreased SCD enzyme activity and mRNA levels in the second study. The ability of TVA to influence SCD activity, therefore, may be associated with dietary energy intake (restricted versus unrestricted food intake in experiments 1 and 2). Results of the third experiment demonstrated that both CLA isomers depressed SCD gene transcription in mouse mammary epithelial cell cultures, but the extent of the reduction was greater in response to 10/12CLA. The 9/11CLA, in contrast, did not depress SCD transcription in bovine cell cultures. This may open up opportunities for increasing 9/11CLA content of bovine milk fat without adversely affecting SCD activity in the mammary gland.

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APPENDIX

TABLE 1 Activities¹ of β -galactosidase in response to fatty acid supplementation in MME cells transfected with reporter plasmids

Fatty acid ²	pCAT-Basic	pCAT-Promoter	pCAT-SCD	SEM ³
SA-0	28.84 ^{a4}	31.68 ^a	16.57 ^a	
SA-50	15.87 ^b	15.75 ^b	5.16 ^b	1.49
SA-100	16.44 ^b	15.82 ^b	8.67 ^b	
OA-0	8.97 ^a	9.42 ^a	11.08 ^a	
OA-50	9.40 ^a	10.67 ^a	8.42 ^a	1.24
OA-100	4.74 ^b	4.38 ^b	8.00 ^a	
TVA-0	25.13 ^a	22.04 ^a	18.38 ^a	
TVA-50	12.73 ^b	17.24 ^a	10.85 ^b	1.63
TVA-100	10.93 ^b	12.33 ^b	9.46 ^b	
9/11CLA-0	18.83 ^a	16.28 ^a	11.33 ^a	
9/11CLA-50	10.81 ^b	12.96 ^{a,b}	7.18 ^b	1.23
9/11CLA-100	7.12 ^c	11.60 ^b	7.23 ^b	
10/12CLA-0	30.00 ^a	26.67 ^a	14.67 ^a	
10/12CLA-50	9.08 ^b	8.67 ^b	22.10 ^a	2.86
10/12CLA-100	12.00 ^b	7.33 ^b	16.43 ^a	
LA-0	29.33 ^a	30.67 ^a	22.46 ^a	
LA-50	12.32 ^b	14.69 ^b	18.31 ^a	3.18
LA-100	9.01 ^b	9.44 ^b	17.89 ^a	
LAN-0	8.50 ^a	16.84 ^a	8.33 ^b	
LAN-50	8.00 ^a	12.12 ^b	8.17 ^b	1.53
LAN-100	5.95 ^a	10.99 ^b	19.94 ^a	

¹ Nanomoles of ONPG (ortho nitrophenyl β -D-galactopyranoside) hydrolyzed per minute.

² SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

³ Pooled SEM.

⁴ Different superscripts (a, b, c) indicate significant differences at $P < 0.05$ due to concentration of a fatty acid within a plasmid.

TABLE 2 Activities¹ of β -galactosidase in response to fatty acid supplementation in Mac-T cells transfected with reporter plasmids

Fatty acid ²	pCAT-Basic	pCAT-Promoter	pCAT-SCD	SEM ³
SA-0	11.46	24.82 ^{a4}	11.51	
SA-50	12.42	13.61 ^b	7.26	3.46
SA-100	11.68	26.99 ^a	10.06	
OA-0	21.93	27.36 ^a	17.93 ^a	
OA-50	17.87	16.41 ^b	10.66 ^b	1.71
OA-100	17.54	13.83 ^b	13.25 ^{a,b}	
TVA-0	10.86 ^a	14.89 ^b	7.93	
TVA-50	15.50 ^b	16.29 ^{a,b}	7.48	0.88
TVA-100	15.51 ^b	17.72 ^a	9.07	
9/11CLA-0	11.46	12.76	5.53	
9/11CLA-50	12.73	14.70	7.02	2.70
9/11CLA-100	15.72	14.77	5.92	
10/12CLA-0	7.37 ^a	10.28 ^b	8.41 ^b	
10/12CLA-50	13.55 ^b	14.31 ^a	15.16 ^a	1.17
10/12CLA-100	18.70 ^c	12.76 ^{a,b}	13.16	
LA-0	9.39 ^a	14.58 ^a	10.28	
LA-50	13.51 ^b	11.25 ^{a,b}	10.14	1.17
LA-100	17.44 ^c	10.64 ^b	9.32 ^a	
LAN-0	14.15 ^a	13.07	8.96	
LAN-50	11.93 ^{a,b}	10.25	8.83	1.13
LAN-100	10.47 ^b	12.51	8.24	

¹ Nanomoles of ONPG (ortho nitrophenyl β -D-galactopyranoside) hydrolyzed per minute.

² SA = stearic acid, OA = oleic acid, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, 10/12CLA = *trans*10,*cis*12-18:2, LA = linoleic acid, and LAN = linolenic acid.

³ Pooled SEM.

⁴ Different superscripts (a, b, c) indicate significant differences at $P < 0.05$ due to concentration of a fatty acid within a plasmid.

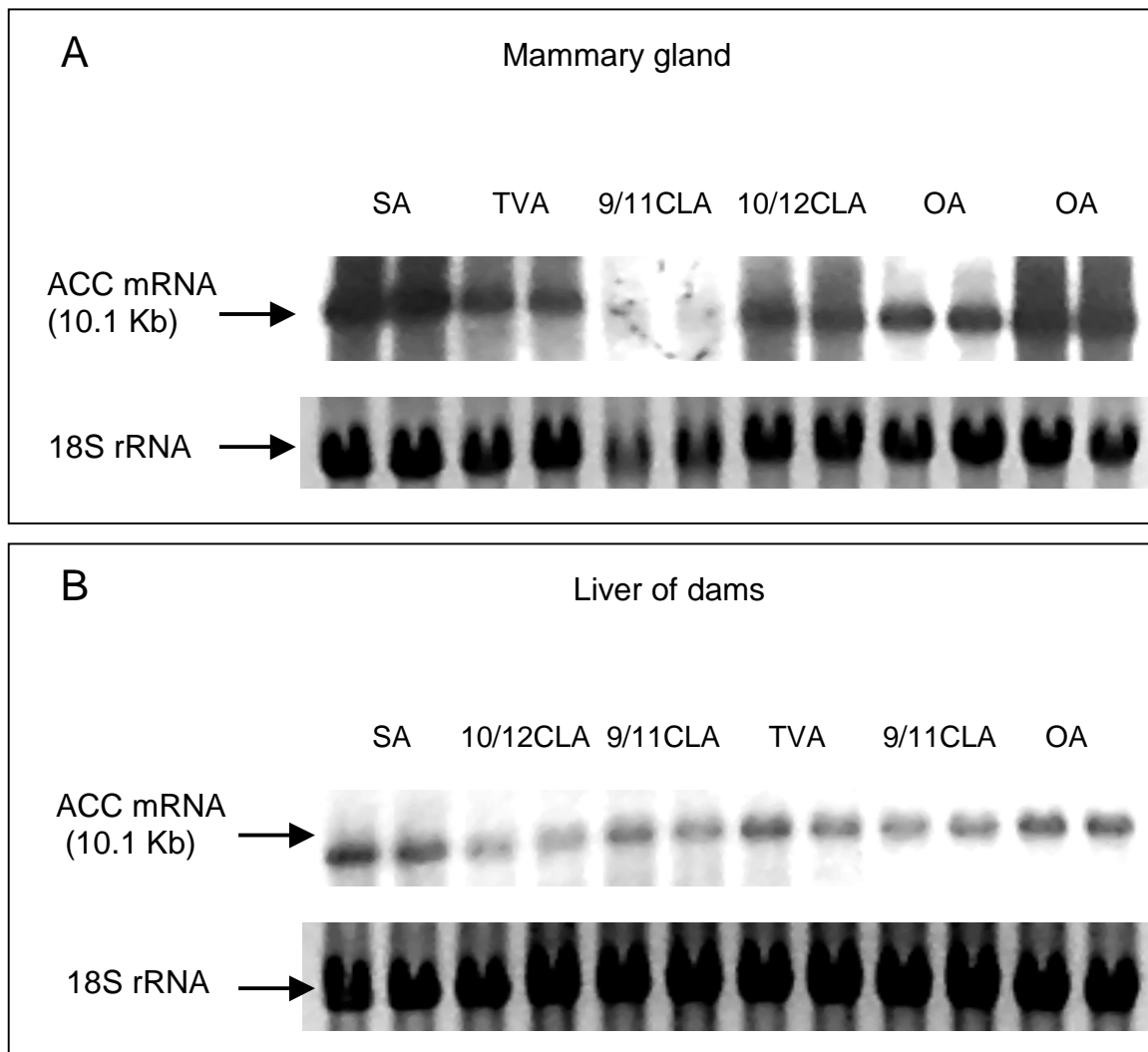


FIGURE 1

Effect of fatty acid supplementation on acetyl-CoA carboxylase (ACC) mRNA abundance in mammary (A) and liver (B) of dams. Samples from each treatment group were in duplicate. SA = stearic acid, OA = control, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, and 10/12CLA = *trans*10,*cis*12-18:2.

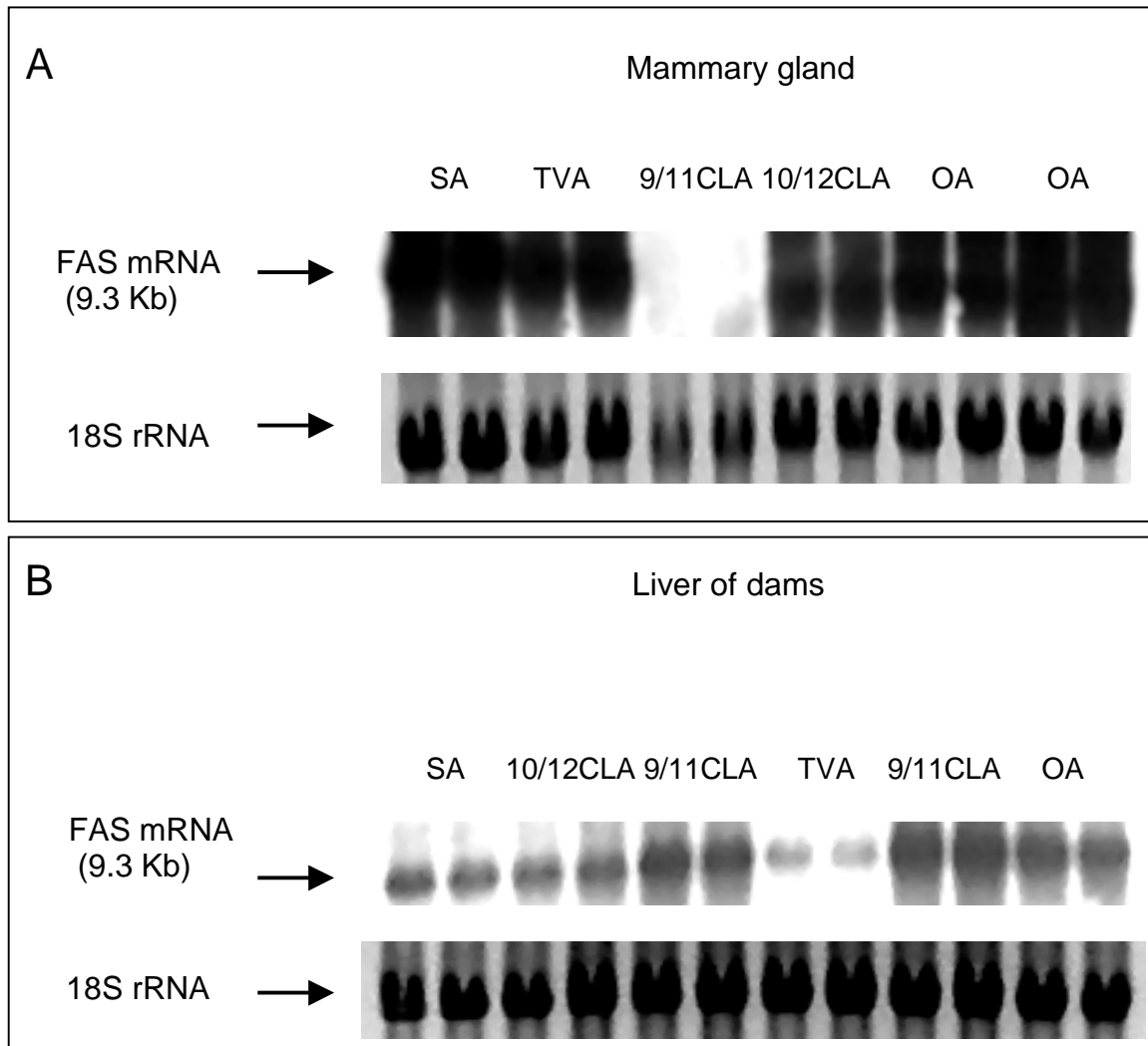


FIGURE 2

Effect of fatty acid supplementation on fatty acid synthase (FAS) mRNA abundance in mammary (A) and liver (B) of dams. Samples from each treatment group were in duplicate. SA = stearic acid, OA = control, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11 18:2, and 10/12CLA = *trans*10,*cis*12-18:2.

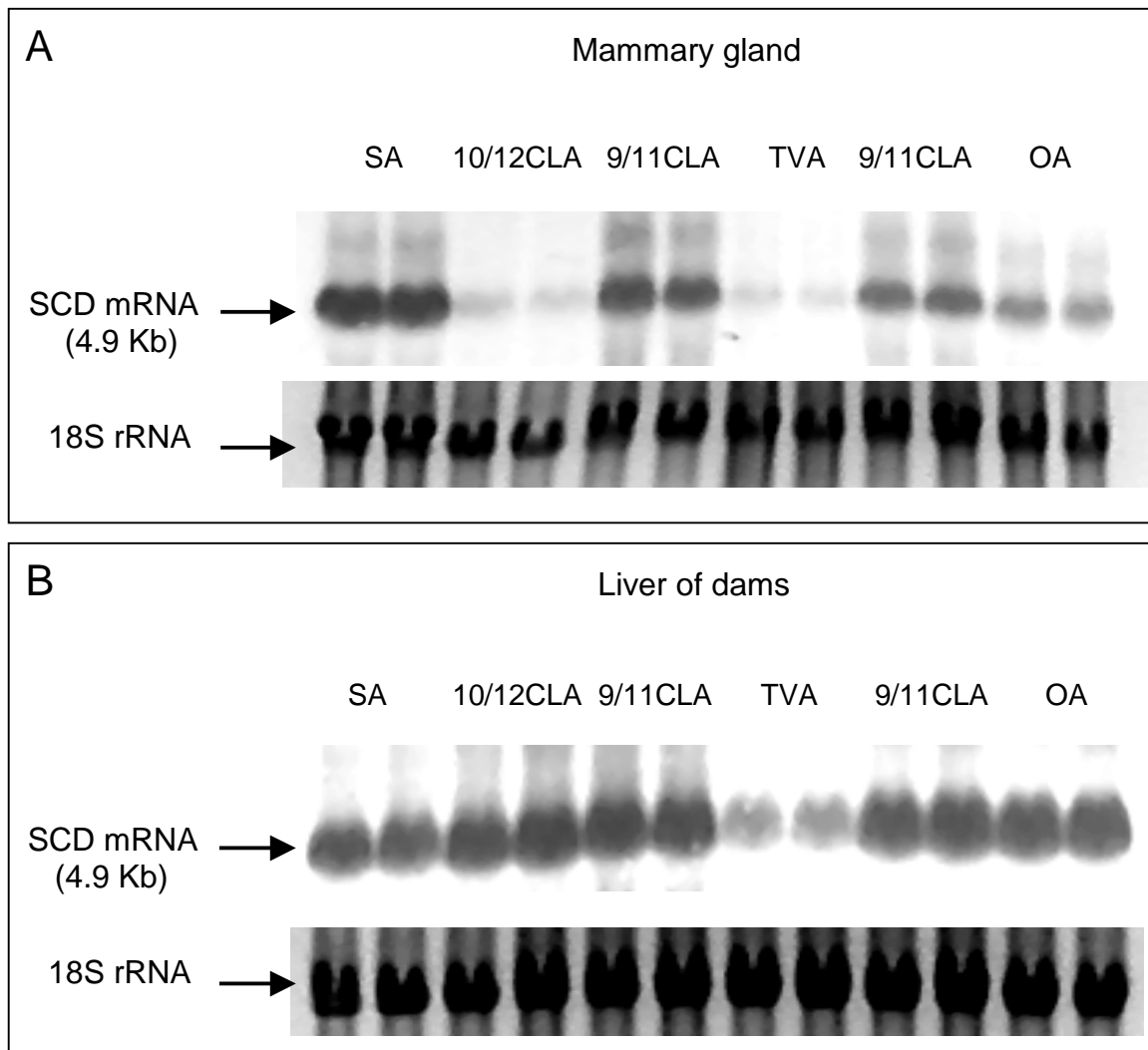


FIGURE 3

Effect of fatty acid supplementation on stearoyl-CoA desaturase (SCD) mRNA abundance in mammary (A) and liver (B) of dams. Samples from each treatment group were in duplicate. SA = stearic acid, OA = control, TVA = *trans*-vaccenic acid, 9/11CLA = *cis*9,*trans*11-18:2, and 10/12CLA = *trans*10,*cis*12-18:2.

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