CHAPTER 4

INFLUENCE OF EXOGENOUS UNSATURATED FATTY ACIDS ON *DE NOVO* FATTY ACID SYNTHESIS IN BOVINE MAMMARY EPITHELIAL CELL CULTURES

Abstract

A preliminary study on the influence of exogenous fatty acids on *de novo* fatty acid metabolism in mouse mammary epithelial cells indicated that *cis* and *trans* isomers of 18:1 and 18:2 produced concentration-dependent effects on cellular fatty acid metabolism. However, it is not known whether bovine mammary epithelial cells would respond to exogenous fatty acids in the same way as mouse mammary cells. The present study was conducted in bovine mammary epithelial cells (MacT) in order to examine the effects of *cis* and *trans* isomers of 18:1 and 18:2 on fatty acid synthesis and desaturation in these cells. MacT cells were plated on uncoated plastic Petri dishes and grown to confluence using Dulbecco's modified Eagle's medium. Media containing supplemental fatty acids bound to bovine serum albumin were added daily to the cells for 3 days. The basal medium contained 100 μM stearic acid (18:0), to which 25, 50 or 100μM of stearic acid, oleic acid, *trans*-vaccenic acid, linoleic acid, or conjugated linoleic acid were added. Cellular responses to the unsaturated isomers were compared with those for 18:0. Cellular fatty acid profiles indicated linear uptake of all the treatment fatty acids by MacT cells. When compared with 18:0, all unsaturated fatty acids reduced cellular 16:0 content, indicating reduced cellular *de novo* fatty acid synthesis. Inhibition of *de novo*

fatty acid synthesis was further supported by the finding that when compared with 18:0, all other treatments inhibited the activities of cellular acetyl-CoA carboxylase and fatty acid synthetase. Results also indicated that the 18:1 and 18:2 isomers were capable of inhibiting transcription of genes for acetyl-CoA carboxylase and fatty acid synthetase. The 18:1 and 18:2 isomers were also capable of modulating enzyme activity and gene expression of stearoyl-CoA desaturase. Conjugated linoleic acid and *trans*-vaccenic acid enhanced activity of desaturase enzyme and increased the abundance of desaturase mRNA in a concentration-dependent manner. Data indicated depression of *de novo* saturated fatty acid synthesis in the mammary gland by *cis* and *trans* isomers of 18:1 and 18:2, and a simultaneous enhancement of Δ^9 desaturase activity by *trans*-vaccenic acid and conjugated linoleic acid.

Introduction

Milk fatty acids are derived from two major sources, from blood and from *de novo* synthesis within the mammary epithelial cells. Each source contributes to approximately 50% of the total milk fatty acid content. It is reasonable to hypothesize that any factor that is capable of modulating the nature of fatty acids in the circulation or capable of modulating *de novo* fatty acid synthesis within the mammary gland would also modulate the composition and content of milk fat. Dietary fat is the major source of fatty acids in circulation. Studies have indicated that it is possible to alter the content and composition of milk fatty acids by supplementing the diet of a dairy cow with certain unsaturated fatty acids. Wonsil et al. (1994) reported that dietary *trans*-vaccenic acid (*trans*-18:1) reduced the percentage of milk fat in cows. Loor et al. (1998) found that dietary soybean oil, which is a rich source of linoleic acid, increased the levels of linoleic (18:2), conjugated linoleic (*cis*, *trans*-18:2) and *trans*-vaccenic acids in the milk fat of Jersey cows. Increasing the conjugated linoleic acid content of milk fat would certainly be beneficial because it is an unsaturated fatty acid, and also due to its potential anticarcinogenic properties (Parodi, 1994). Jenkins (1998) observed an increase in the oleic acid (18:1) content and a decrease in the palmitic acid (16:0) content of cow's milk consequent to dietary supplementation of rumen-protected oleic acid.

The mechanism by which dietary fat is able to bring about changes in milk fat composition is not clear. Studies at the cellular level are necessary to understand the influence of external fatty acids on fatty acid metabolism within the mammary epithelial

cells. A preliminary study (Jayan et al., 1997) using mouse mammary epithelial cells indicated that exogenous *cis* and *trans* isomers of 18:1 and 18:2 modulated the activities of mammary fatty acid synthetase and stearoyl-CoA desaturase. Differences exist between murine and ruminant species in some of the pathways involved in fatty acid biosynthesis. There is evidence that even within the same species the effect of exogenous fatty acids on cellular fatty acid biosynthesis is tissue-specific (Kim and Freake, 1996). Moreover, it is not known whether bovine mammary cells would respond to exogenous fatty acids in the same way as murine mammary cells. The present study was conducted using MacT cells to understand the influence of *cis* and *trans* isomers of 18:1 and 18:2 on activity and mRNA abundance of acetyl-CoA carboxylase, fatty acid synthetase and stearoyl-CoA desaturase.

Materials and Methods

Cell culture

Bovine mammary epithelial cells (MacT cells) were plated on uncoated plastic Petri dishes (Corning, NY). The cells were grown to a confluent monolayer using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, MO). The DMEM was supplemented with 14.3 mM sodium bicarbonate and 3.1 mM sodium acetate then pH was adjusted to 7.4. The medium was also supplemented with 100 mL/L fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta), 10 mg/L apo-transferrin (Atlanta Biologicals), 5000 U/L penicillin (Sigma), 5 mg/L streptomycin (Sigma), and 10 mg/L gentamicin (Sigma). The complete medium mixture was filter-sterilized through 0.2 μ surfactant free cellulose acetate (SFCA) filters (Nalgene, NY).

Treatment media were supplemented with treatment fatty acids (FA). The treatments are shown in Table 4.1. 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:FA was 1:3. Media containing supplemental FA were filter sterilized through 0.45 μ SFCA filters.

Treatment media were applied to confluent cell cultures and changed daily for three days. At the end of the three-day treatment period, cells were collected for analysis.

Cell collection

Media was removed from the plates, cells were rinsed with phosphate buffered saline (PBS), and the cells were then scraped into 5 mL PBS added to the plate. The mixture was transferred to a sterile plastic centrifuge tube and the cells were pelleted by centrifugation at 1200 x g for 5 min. The PBS contained 137 mM NaCl, 2.7 mM KH_2PO_4, and 10 mM Na_2HPO_4.

A total of 30 plates were used for each treatment, and from this triplicate observations were made for each response. Three sets of three plates each were taken for cellular FA analysis by gas chromatography. For this, the cell pellets from three plates each were combined and transferred to a glass screw capped tube, and centrifuged at 1200 x g for 5 min. The supernatant was discarded and the cells were frozen at -20^{o}C until analysis. Three sets of three plates each were taken for estimation of protein and DNA contents and activities of acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS) and stearoyl-CoA desaturase (SCD). Each plate was resuspended in 1 mL PBS and stored at -70^{o}C until analysis. Twelve plates from each treatment were taken for extraction of total RNA and Northern blot analysis of ACC, FAS and SCD mRNA abundance. Total RNA was immediately extracted from the plates, and the RNA samples were stored at -80^{o}C.

Cellular DNA estimation

Cellular DNA content in the crude cell homogenates was determined using the fluorometric method described by Labarca and Paigen (1980). It was based on the enhancement of fluorescence when bisbenzimidazole (Hoechst 33258) binds to DNA.

The cells were homogenized in phosphate-saline buffer (0.05 M Na_2HPO_4 and 2 M NaCl, pH 7.4) and sonicated for 10 seconds in order to dissociate chromatin. Aliquots of the homogenate were then mixed with phosphate-saline buffer containing compound Hoechst 33258 to a final concentration of 1 μg/mL. Fluorescence of the samples were then measured using a fluorocolorimeter. The DNA contents in the samples were calculated from a standard curve plotted using fluorocolorimetric measurements of known concentrations of standard DNA.

Cell protein determination

Cellular protein content was determined using the spectrophotometric Pierce Bicinchoninic Acid Protocol (Pierce, IL). The microtiter plate assay protocol, as described by the reagent manufacturer was used. Absorbance was read at 560 nm, on a Titertek Multiscan MMC/340 microtiter plate reader (Eplab, Finland). The protein contents in the samples were calculated from a standard curve plotted using spectrophotometric measurements of known concentrations of standard protein. Basically, the assay protocol combines the reaction of protein with Cu^{++} in an alkaline medium to form Cu^{+}. The highly sensitive and selective reagent for detection of Cu^{+} was bicinchoninic acid (BCA). The purple reaction product formed by the interaction of two molecules of BCA with one cuprous ion is water-soluble and exhibits strong absorbance at 562 nm. This allows spectrophotometric quantitation of protein in aqueous solution.

FA analysis

Methyl esters of cellular FA were prepared by *in situ* transesterification, according to the base-catalyzed methylation procedure of Park and Goins (1994), and using 11:1 (undecenoic acid) as the internal standard for peak quantification in the gas chromatogram. Samples were split 20:1 and injected by an auto sampler into a 30 m SP2380 fused silica capillary column of a Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett Packard Co., CA). Injector and detector temperatures were set at 225^{o}C. A temperature program initiated runs at 60^{o}C and warmed gradually to 225^{o}C. Identification of sample peaks was based on relative retention times of a commercial standard (Nu-Chek Prep, MN). The FA in the standard were used to construct a calibration table using a Hewlett Packard 3396A integrator (Hewlett Packard Co., PA). Response and recovery factors were used to determine weight percentages of individual FA in the cell samples. Finally, the cellular FA content was expressed on a per mg protein basis.

ACC assay

ACC activity in the bovine cells was assayed according to the bicarbonate fixation protocol of Nakanishi and Numa (1970), which follows the rate of acetyl-CoA-dependent incorporation of ^{14}C-bicarbonate into acid-stable malonyl-CoA. The samples were first pre-incubated at 37^{o}C for 30 min. 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 0.75 mg/mL bovine serum albumin, in a final volume of 1 mL. At the end of 30 min, reaction was started by the addition of 0.8 mL of assay mixture containing 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, 3.75 mM bovine serum albumin and 12.5 mM $KH^{14}CO_3$ (50, 000 CPM). The reaction was allowed to proceed for 15 minutes at 37^{o}C and then terminated with 0.2 mL of 5 N HCl. It was then centrifuged at 1000 rpm for 10 minutes, the supernatant was collected into a scintillation vial, scintillation fluid was added and the radioactivity was determined using a liquid scintillation counter. The amount of malonyl-CoA formed was calculated form the radioactivity and the specific activity of ^{14}C-bicarbonate. Finally ACC activity was expressed as amount of malonate formed per mg cell protein.

FAS assay

Cellular FAS activity was measured according to the procedure of Smith and Abraham (1970). The assay system contained 0.1 M potassium phosphate (Na_2HPO_4) pH 6.6, 1 mM DTT (dithiothreitol), 0.15 mM NADPH and 0.05 mM acetyl-CoA. After incubating the mixture at 30^{o}C for 5 minutes, the reaction was initiated by the addition of 55 μM 2-^{14}C-malonyl-CoA (50, 000 CPM). Final assay volume was 0.5 mL. The reaction was allowed to proceed for 30 min at 30^{o}C, and then it was terminated by the addition of 0.1 mL of 30% aqueous KOH. The mixture was heated to 80^{o}C, cooled on ice and acidified with 0.3 mL of 4 M HCl. The FA formed were extracted three times, each with 2 mL hexane. The hexane was then evaporated under nitrogen, scintillation fluid was added, and the radioactivity was measured using a liquid scintillation counter. The amount of FA formed (which would be primarily palmitate) was calculated from the radioactivity and specific activity of malonyl-CoA. Finally, FAS activity was expressed as the amount of palmitate formed per mg cell protein.

SCD assay

Cellular Δ^9desaturase activity was measured according to the protocol of Cameron et al. (1994). The reaction mixture contained 1 mM NADPH, 0.1 M Tris-HCl pH 7.25 and 70 μM 1-^{14}C-stearoyl-CoA (50,000 CPM), in a final volume of 0.5 mL. The reaction was allowed to continue under aerobic conditions for 20 min at 37^{o}C, and then terminated with 0.5 mL of 10% KOH in methanol. It was then heated at 90^{o}C for 25 minutes, cooled to room temperature and acidified with 0.5 mL of 4 M HCl. The FA formed were extracted 3 times, each with 2 mL hexane. The hexane was then evaporated under nitrogen, and the FA were methalyted using 14% BF_3 in methanol. The stearate and oleate methyl esters were then separated on 10% $AgNO_3$-coated glass silica gel plates using hexane:diethylether (9:1) as the solvent system. The spots were visualized by spraying with water, and comparing to known standards. The spots corresponding to oleate were scraped into scintillation vials, and the radioactivity was measured in a liquid scintillation counter after the addition of scintillation fluid. From the radioactivity, the amount of oleate formed was calculated using specific activity of stearoyl-CoA. The SCD activity was expressed as the amount of oleate formed per mg cell protein.

Extraction of total RNA

Total RNA from the cells was extracted using TriReagent, following the manufacturer's (Molecular Research Center Inc., OH) protocol. The RNA was then dissolved in DEPC-treated water and stored at -80^{o}C. Purity of the RNA was judged by the ratio of spectrophotometric $Absorbance_{260}/Absorbance_{280}$, and RNA was quantitated using $Absorbance_{260}$.

Northern blotting and assay of mRNA abundance

Total RNA (30 μg) was subject to electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde. After electrophoresis, RNA was visualized by ethidium bromide staining and photographed using Polaroid 55 positive/negative film. RNA on the gel was then transferred to nylon Magna membrane by downward capillary action using 10X SSC as the transfer buffer. The 10X SSC contained 1.5 M NaCl and 2 M sodium citrate, pH 7. The RNA on the membrane was cross-linked by ultraviolet irradiation at 1200 $\mu W/cm^2$ for 3 min. Subsequently, membranes were prehybridized and then hybridized with 1 x 10^6 cpm/mL ^{32}P-labelled cDNA probe specific for the mRNA (ACC, FAS or SCD). Concentration of probe in the hybridization solution was 10 ng/mL. Each probe was hybridized to 3 membranes, so that triplicate observations could be obtained for statistical analysis. The prehybridization/hybridization solution contained 50% formamide, 5X SSPE pH 7.4, 5X Denhardt's, 0.2% SDS and 10 μg/mL tRNA. The 5X SSPE contained 0.75 M NaCl, 0.05 M Na_2HPO_4, and 5 mM EDTA. The 5X Denhardt's solution contained 0.1% polyvinyl pyrrolidone-360, 0.1% ficoll-400 and 0.1% bovine serum albumin.

After hybridization, the membranes were washed at medium stringency (*ie.* the membranes were washed twice for 15 min each in 5X SSPE containing 0.5% SDS at room temperature, then twice for 15 min each in 1X SSPE containing 0.5% SDS at 37^{o}C, and finally in 0.1X SSPE containing 1% SDS for 15 min at 37^{o}C). Subsequently, binding of the labeled probe to target RNA was visualized by autoradiography of the hybridized membrane on a Kodak X-Omat film with two intensifying screens at -80^{o}C. In order to

ensure equivalency of loading of RNA on the gel, and to quantitate the mRNA bands, the membranes were reprobed with a labeled β-actin probe, after stripping it with 0.1X SSC containing 1% SDS. Again, binding of the β-actin probe was visualized by autoradiography of the hybridized membrane on a Kodak X-Omat film. Then the intensity of all the mRNA bands were quantitated by laser densitometry scanning. The abundance of each mRNA in question (ACC, FAS or SCD) was expressed as the ratio of density of that mRNA band to the density of β-actin mRNA band. Autoradiogram of Northern blot hybridized with probe for SCD mRNA is shown in Appendix I.

The probes used for Northern blot analysis were kindly donated by the following persons: probe for SCD by Dr. James Ntambi, University of Wisconsin, Madison; probe for FAS by Dr. Mark Magnuson, Vanderbilt University Medical Center, Nashville; and probe for ACC by Drs. Mike Barber and Maureen Travers, Hannah Research Institute, Scotland. Sufficient quantities of the probe were prepared (Appendix I) for use in Northern blot analyses.

Statistical analysis

All data were analyzed by the general linear model procedure of SAS (1985) (Appendix II). A two-way analysis of variance model was used.

$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + E_{ijk}$

The two factors in the model were treatment fatty acid and treatment concentration. Y_{ijk} is the response, A_i is the treatment fatty acid effect, B_j is the effect of treatment concentration, $(AB)_{ij}$ is the fatty acid by concentration interaction effect, E_{ijk} is the

residual error and μ is the mean. Individual means were compared by Tukey's means comparison procedure, and considered significantly different at $P < 0.05$. Within each treatment fatty acid, the nature of cellular response (whether significant linear or significant quadratic) with increasing treatment concentration was tested by regression analysis. The model used was $Y = \beta_0 + \beta_1 X_i + \beta_2 X_i + E_i$, where Y is the cellular response to treatment X_i, β_0 is the Y-intercept of the regression line, β_1 is the linear regression coefficient, β_2 is the quadratic regression coefficient and E_i is the error. Responses were considered significant linear or significant quadratic at $P < 0.05$.

Results and Discussion

Cell protein and DNA contents

Protein and DNA contents in MacT cells in response to fatty acid (FA) treatment are given in Table 4.2. Cellular protein and DNA contents are indicators of cell number and cell size. However, in this experiment the protein and DNA contents were measured in order to express the other cellular parameters on a protein or DNA basis.

Cellular FA profile

The FA content in MacT cells in response to external FA treatment is given in Table 4.3. There was a positive relationship between media concentration of a treatment FA, and its concentration in the cells (Figure 4.1). This indicated that the cells were capable of extracting and incorporating the treatment FA. Hence, it is possible that the treatment FA could affect FA metabolism within the cells. The probable ways by which external FA could interact with cellular *de novo* FA synthesis are indicated in Figure 4.2.

Cellular 16:0 content

The media did not contain 16:0, but acetate which is the precursor for cellular 16:0 synthesis was provided in the media. So, the most likely source of 16:0 in the MacT cells was from *de novo* synthesis. Hence, the cellular 16:0 content can be taken as an indicator of *de novo* FA synthesis by the cells.

Cellular 16:0 contents in response to FA treatment are shown in Figure 4.3. Stearic acid (SA) caused a concentration-dependent linear increase in cellular 16:0 content. This indicates that SA enhanced *de novo* FA synthesis by the cells. A higher rate of *de novo* FA synthesis by SA treatment could be due to enhanced activities of cellular acetyl-CoA carboxylase (ACC) and/or fatty acid synthetase (FAS). All other treatments tended to reduce cellular 16:0 content with increasing concentration, suggesting inhibition of *de novo* FA synthesis by the cells. In an earlier study conducted in our lab, Dawson and Herbein (1996) observed that 18:3 and conjugated linoleic acid (CLA) inhibited *de novo* synthesis of 16:0 in MacT cells, in a concentration-dependent manner.

Cellular 18:0 content

18:0 content in MacT cells increased with increase in concentration of SA in the media (Table 4.3). Cellular 18:0 content also increased as the concentration of linoleic acid (LA) in the treatment media increased from 25 to 50 μM, and as the concentration of oleic acid (OA) increased from 50 to 100 μM. This could be a result of enhanced uptake of 18:0 from the media, because all media contained 100 μM 18:0. It has been reported that FA modulate cellular functions by affecting membrane fluidity, and the ratio of the amount of saturated to unsaturated FA in cell membranes is essential to maintain normal physiological membrane fluidity (Merrill and Schroeder, 1993). Incorporation of excess unsaturated LA or OA into the cell membranes could increase their fluidity and may disrupt their normal physiological status. In order to compensate for this disruption, it is reasonable that the cells would take up more of the available 18:0 from the media. CLA

reduced cellular 18:0 content at 50 and 100 μM, and this could be due to inhibition of 18:0 uptake by CLA.

Cellular *cis*-16:1 content

The media did not contain *cis*-16:1. The most likely sources of *cis*-16:1 in the cells are desaturation of the *de novo* synthesized 16:0, or retroconversion of *cis*-18:1. Increasing the concentration of SA from 25 to 50 μM increased cellular *cis*-16:1 content (Table 4.3). Cellular 16:0 content increased with increase in treatment SA concentration. So, the increase in *cis*-16:1 in response to treatment with SA could be due to increased rate of desaturation of 16:0 to *cis*-16:1 as a result of increased substrate availability and/or due to increased activity of the desaturase enzyme. All other treatment FA tended to lower cellular *cis*-16:1 with increasing treatment concentrations. These treatments also lowered cellular 16:0 content. Hence, the lowering of cellular *cis*-16:1 content could be due to reduced substrate availability or due to inhibition of desaturase activity.

Cellular *trans*-16:1 content

From Table 4.3 it can be seen that only the cells treated with *trans*-vaccenic acid (TVA) contained detectable amounts of *trans*-16:1. There was a significant linear relationship between cellular *trans*-16:1 and the concentration of TVA in the treatment media. Neither *cis*- nor *trans*-16:1 was supplemented in the media, and the product of 16:0 desaturation is *cis*-16:1. So, the most likely source of *trans*-16:1 in the cells would be incomplete peroxisomal β-oxidation of TVA isomer. Retroconversion of saturated and unsaturated FA has been reported earlier in several cell types, though not in mammary

cells. Bourre et al. (1982) observed retroconversion of elaidic acid to *trans*-16:1 in mouse sciatic nerve cell cultures, and Panigrahi and Sampugna (1993) reported retroconversion of *trans*-18:1 isomers to *trans*-16:1 isomers in Swiss mouse fibroblast cells. The present study indicates the presence of active retroconversion in bovine mammary epithelial cells.

Activities of ACC, FAS and SCD

Cellular ACC activity

SA caused a concentration-dependent increase in cellular ACC activity. All other treatments, *viz*. OA, TVA, LA and CLA caused concentration-dependent inhibition of the enzyme activity (Figure 4.4). At all treatment concentrations, SA significantly increased cellular ACC activity, when compared with the other treatments. The most significant inhibition of ACC activity at all treatment concentrations was produced by TVA and CLA, followed by LA and OA (Table 4.4). The significance of these findings will be discussed along with the findings for cellular FAS activity in response to FA treatment.

Cellular FAS activity

FAS activity in MacT cells in response to FA treatment is shown in Figure 4.5. Response in FAS activity was similar to that observed for ACC activity. SA enhanced the enzyme activity in a concentration-dependent manner, whereas all other treatments inhibited FAS activity in a concentration-dependent manner. At all treatment concentrations, SA significantly increased the enzyme activity when compared with the other treatments.

The most significant inhibition was caused by TVA and CLA, followed by OA and LA (Table 4.4).

The observations for ACC and FAS activities in MacT cells indicate that SA enhanced *de novo* FA synthesis by the cells. In the present study, it was also seen that SA caused a concentration-dependent increase in cellular 16:0 content (Table 4.3). This increase in 16:0 content could be a result of increased ACC and FAS activities, leading to enhanced rate of conversion of acetate to 16:0.

There has been previous reports suggesting that dietary unsaturated FA were potential inhibitors of FA biosynthesis and that saturated FA were enhancers of FA biosynthesis in various tissues. As early as 1969, Allmann and Gibson discovered that by adding 2% LA to a high-carbohydrate, fat-free diet of mice, the rate of hepatic FAS activity was depressed by 70% in 2 days. Strum-Odin et al. (1987) found that FAS activity increased in hepatocytes conditioned to media containing 16:0. Results of the present study in mammary MacT cells are in agreement with these earlier findings.

Results of this study could also explain milk fat depression caused by dietary intake of certain unsaturated fatty acid isomers in ruminants and non-ruminants. Selner and Schultz (1980) reported that dietary *trans*-FA caused milk fat depression in lactating cows, and Teter et al. (1990) reported that dietary *trans*-FA depressed the percentage of fat in mouse milk. Similarly, Wonsil et al. (1994) observed an inverse relationship between the percentage of milk fat and duodenal flow of *trans*-18:1. Also, Loor and

Herbein (1997) observed that abomasal infusion of CLA reduced milk fat percentage in Holstein cows. None of the above studies investigated the mechanisms involved in alteration of milk fat composition by dietary FA. The findings of the present study suggest that these effects were a consequence of inhibition of cellular ACC and FAS activities in the mammary tissue.

Alteration of ACC and FAS activities can be brought about by several ways. ACC can be regulated by allosteric control mechanisms, by phosphorylation, or by regulation of synthesis and degradation of the protein (Kim, 1997). FAS activity is also subject to regulation by dietary factors, but the mechanisms involved have not been defined. Paulauskis and Sul (1988) suggested that the most likely way in which hepatic FAS activity was regulated by dietary and hormonal factors was by altering the concentration of the enzyme, by regulating either its rate of synthesis from the mRNA, or its rate of degradation.

Cellular SCD activity

SCD activity in MacT cells in response to FA treatment is shown in Figure 4.6. Increasing the concentration of treatment-SA had very little effect on the enzyme activity. When compared with SA, TVA and CLA increased cellular SCD activity at all concentrations, whereas OA reduced the enzyme activity. At all treatment concentrations, SCD activity in response to CLA and TVA was significantly higher than that of the other treatments (Table 4.4).

In eukaryotes, saturated FA are precursors of unsaturated FA, and introduction of the first *cis*-double bond at the Δ^9 position by SCD is a critical step in the synthesis of unsaturated FA from saturated FA. It was seen earlier that cellular *cis*-16:1 content in MacT cells was increased by SA, whereas it was decreased by the other treatments. So, a similar response in SCD activity was expected. Also, incorporation of SA into the cells should have triggered desaturase enzyme activity, as a physiological response to maintain normal membrane fluidity. However, it is seen that SCD activity did not follow the expected pattern in response to SA treatment. Due to the same reason of maintaining normal membrane fluidity, it would be reasonable to expect reduced SCD activity in response to unsaturated FA treatment. In the present study, the expected response was observed for LA and OA. TVA is a *trans* isomer and it could possibly behave more like a saturated FA than like an unsaturated FA. So, the increase in desaturase activity by TVA could be a physiological response to maintain membrane fluidity. It was also observed in this study that, when compared with the control and SA-treatment, CLA enhanced desaturase activity. CLA is *cis, trans*-18:2. It is possible that CLA is competing with LA (which is *cis, cis*-18:2) for incroporation into cell membranes. Having one of the double bonds in *trans* configuration would make CLA less fluid in nature when compared with LA. Increase in SCD activity by CLA could be a cellular response to synthesize greater amounts of *cis* unsaturated fatty acids for incorporation into cell membranes in order to maintain normal membrane fluidity.

Similar responses in SCD activity in tissues other than the mammary gland have been reported previously. Egwin and Kummerow (1972) found that desaturase activity in rat liver was inhibited by dietary 18:1 and 18:2. Cook (1981) found that dietary oleic acid inhibited desaturase activity in rat brain, whereas *trans*-vaccenic acid and *trans, trans*-18:2 stimulated the enzyme activity.

In the present study, investigation was done only on the activities of cellular ACC, FAS and SCD. No attempt was made to measure the cellular content of these enzymes. So, it is not possible to infer whether the differences in enzyme activities were due to differences in the corresponding protein contents or not. Further investigation is necessary to explain the cause of altered enzyme activity in response to FA treatment.

Abundance of ACC, FAS and SCD mRNA

Cellular ACC and FAS mRNA abundance

Abundance of cellular ACC and FAS mRNA in response to FA treatment followed the same pattern as that of the respective enzymes. ACC and FAS mRNA abundance in response to treatment are shown in Figures 4.7 and 4.8, respectively. SA is the only treatment that elevated mRNA levels of ACC and FAS. When compared with SA, at all concentrations the other treatments significantly reduced the abundance of ACC and FAS mRNA. So, the changes in enzyme activities observed could have been a result of changes in the enzyme concentration arising from changes in availability of substrate mRNA for protein formation.

Previous reports indicated unsaturated FA suppressed transcription of lipogenic genes, when compared with saturated FA. Cheema and Clandinin (1996) reported that when compared with a saturated-fat rich diet consumption of a diet high in polyunsaturated fatty acids significantly decreased the levels of ACC and FAS mRNA in mouse liver. Kasturda et al. (1990a, 1990b) observed that dietary corn oil reduced the rate of transcription and mRNA abundance of ACC and FAS in rat liver.

Cellular SCD mRNA abundance

Response of SCD mRNA abundance to FA treatment was similar to that observed for SCD enzyme activity (Figure 4.9). LA and OA had very little effect on SCD mRNA abundance. When compared with the other treatments, CLA and TVA significantly increased the abundance of desaturase mRNA at all concentrations (Table 4.4). SA tended to increase the mRNA abundance with increasing treatment concentration, but a parallel increase in enzyme activity was not observed. This could be due to some factor that prevented translation of mRNA to the active protein, or due to degradation of the mRNA before it was converted to protein, or probably due to some factor that prevented activation of the protein that has already been formed.

These observations indicate that treatment FA not only affected activity of desaturase enzyme, but modulated desaturase mRNA abundance also. Similar results have been reported in previous studies. Paisley et al. (1996) reported that dietary corn oil reduced the abundance of SCD mRNA in mouse liver. Sessler et al. (1996) found that arachidonic acid, linoleic acid and linolenic acid decreased SCD mRNA abundance in

mature adipocyte cultures, by reducing the half-life of the mRNA. They also reported that stearic and oleic acids did not affect the mRNA abundance.

In the present study, only the abundance of ACC, FAS and SCD mRNA were measured. Abundance of an mRNA is an indicator of its rate of synthesis and degradation, and reflects the half-life of the mRNA. However, differences in mRNA abundance between treatments could be due to various factors such as direct control of gene transcription (Thompson and Towle, 1991), control of mRNA processing (Burmeister and Mariash, 1991), control of mRNA editing (Baum et al., 1990) and/or control of mRNA stability (Dozin et al., 1986). In the present study, the rate of transcription of the desaturase gene, the rate of processing or degradation of the primary transcript, or the actual amount of the protein in response to treatments were not measured. So, it is not possible to conclude whether the responses observed were due to an effect at the gene level or not. Further investigation is necessary to establish the cause of altered mRNA in response to fatty acid treatment.

Table 4.1
Concentration of supplemental fatty acids in treatment media applied to confluent MacT cell cultures.

Treatment*	Supplemental fatty acid in treatment media (μM)				
	SA*	OA*	TVA*	LA*	CLA*
Control	100				
25-SA	125				
50-SA	150				
100-SA	200				
25-OA	100	25			
50-OA	100	50			
100-OA	100	100			
25-TVA	100		25		
50-TVA	100		50		
100-TVA	100		100		
25-LA	100			25	
50-LA	100			50	
100-LA	100			100	
25-CLA	100				25
50-CLA	100				50
100-CLA	100				100

*SA is stearic acid, OA is oleic acid, TVA is *trans*-vaccenic acid, LA is linoleic acid and CLA is conjugated linoleic acid.

Table 4.2
Cellular protein and DNA contents in MacT cells in response to fatty acid supplementation

	Cell fraction (μg per plate) [1]	
Treatment*	DNA	Protein
Control	174.8	261.3
25-SA	186.1[ab]	275.0[b]
25-OA	193.5[a]	348.9[a]
25-TVA	198.5[a]	339.9[a]
25-LA	198.2[a]	358.8[a]
25-CLA	176.6[b]	277.4[b]
50-SA	194.2 [a]	311.3[b]
50-OA	200.9 [a]	362.9[a]
50-TVA	201.5 [a]	359.4[a]
50-LA	199.2 [a]	355.9[a]
50-CLA	193.3 [a]	338.1[a]
100-SA	208.5 [a]	352.3[a]
100-OA	199.2 [a]	361.9[a]
100-TVA	205.0 [a]	377.2[a]
100-LA	200.0 [a]	361.4[a]
100-CLA	192.9 [a]	343.6[a]
Standard Error	3.2	7.1

*SA is stearic acid, OA is oleic acid, TVA is *trans*-vaccenic acid, LA is linoleic acid and CLA is conjugated linoleic acid.

[1]Different superscripts (a, b) indicate significant differences at $P < 0.05$ between fatty acid treatments within the same concentration.

Table 4.3
Cellular fatty acid profile in MacT cells in response to fatty acid treatment

Treatment*	Cellular fatty acid content (μg per mg protein)[1]							
	16:0	*trans* 16:1	*cis* 16:1	18:0	*trans* 18:1 (Δ11)	*cis* 18:1	18:2	CLA
Control	21.2	0	4.5	50.3	0	52.9	8.0	0
25-SA	32.4^{a}	0^{b}	6.3^{b}	69.1^{a}	0^{b}	62.8^{a}	10.8^{b}	0^{b}
25-OA	28.4^{a}	0^{b}	11.6^{a}	42.0^{b}	0^{b}	55.4^{a}	5.8^{b}	0^{b}
25-TVA	23.7^{a}	3.5^{a}	4.0^{bd}	44.1^{b}	9.8^{a}	32.6^{b}	6.0^{b}	0^{b}
25-LA	22.3^{a}	0^{b}	2.0^{d}	46.2^{b}	0^{b}	21.3^{b}	55.0^{a}	0^{b}
25-CLA	27.1^{a}	0^{b}	5.2^{bc}	70.5^{a}	0^{b}	32.2^{b}	10.6^{b}	20.7^{a}
50-SA	48.0^{a}	0^{b}	10.0^{a}	115.0^{a}	0^{b}	112.8^{a}	18.3^{b}	0^{b}
50-OA	15.9^{b}	0^{b}	3.7^{b}	31.4^{c}	0^{b}	51.1^{a}	4.6^{c}	0^{b}
50-TVA	16.7^{b}	6.7^{a}	2.7^{b}	39.4^{c}	17.0^{a}	35.0^{ab}	6.9^{bc}	0^{b}
50-LA	25.1^{b}	0^{b}	2.0^{b}	91.7^{b}	0^{b}	27.0^{b}	117.1^{a}	0^{b}
50-CLA	25.0^{b}	0^{b}	2.5^{b}	44.2^{c}	0^{b}	22.4^{b}	7.3^{bc}	23.7^{a}
100-SA	49.5^{a}	0^{b}	7.5^{a}	153.7^{a}	0^{b}	117.2^{a}	16.5^{b}	0^{b}
100-OA	17.3^{b}	0^{b}	0.9^{c}	52.8^{bc}	0^{b}	90.7^{b}	5.9^{c}	0^{b}
100-TVA	19.2^{b}	13.7^{a}	2.7^{b}	53.8^{bc}	49.9^{a}	41.5^{c}	6.7^{c}	3.8^{b}
100-LA	20.0^{b}	0^{b}	1.4^{c}	76.0^{b}	1.8^{b}	19.1^{cd}	113.5^{a}	0^{b}
100-CLA	18.2^{b}	0^{b}	1.7^{c}	44.2^{c}	0^{b}	14.5^{d}	6.3^{c}	33.3^{a}
Standard Error	2.2	0.3	0.7	4.8	1.9	4.0	2.1	0.8

*SA is stearic acid, OA is oleic acid, TVA is *trans*-vaccenic acid, LA is linoleic acid and CLA is conjugated linoleic acid.

[1]Different superscripts (a, b, c) indicate significant difference at $P < 0.05$, between fatty acid treatments within the same concentration.

Table 4.4
Enzyme activities and mRNA abundance of cellular acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS) and stearoyl-CoA desaturase (SCD) in MacT cells in response to fatty acid treatment

	Enzyme activity[1]			Cellular mRNA[1]		
Treatment*	ACC[2]	FAS[3]	SCD[4]	ACC[5]	FAS[5]	SCD[5]
Control	7.4	3.9	1.3	0.42	0.38	6.5
25-SA	7.5^{a}	4.2^{a}	1.4^{b}	0.46^{a}	0.45^{a}	6.5^{c}
25-OA	6.0^{b}	3.4^{b}	0.9^{b}	0.39^{a}	0.33^{b}	6.4^{c}
25-TVA	4.2^{c}	2.2^{c}	2.3^{a}	0.22^{b}	0.22^{c}	7.9^{b}
25-LA	5.3^{b}	3.2^{b}	1.5^{b}	0.33^{a}	0.28^{bc}	6.5^{c}
25-CLA	5.7^{b}	2.1^{c}	2.8^{a}	0.25^{b}	0.22^{c}	8.3^{a}
50-SA	8.2^{a}	4.7^{a}	1.3^{b}	0.48^{a}	0.49^{a}	6.8^{c}
50-OA	5.9^{b}	2.9^{b}	1.0^{b}	0.38^{b}	0.32^{b}	6.5^{c}
50-TVA	3.3^{c}	1.8^{c}	2.4^{a}	0.20^{c}	0.21^{c}	9.5^{a}
50-LA	4.9^{b}	2.9^{b}	1.2^{b}	0.33^{b}	0.27^{bc}	6.2^{c}
50-CLA	4.2^{b}	1.7^{c}	2.4^{a}	0.21^{c}	0.19^{c}	8.1^{b}
100-SA	8.6^{a}	4.6^{a}	1.2^{b}	0.58^{a}	0.50^{a}	7.9^{b}
100-OA	5.4^{b}	2.8^{b}	1.0^{b}	0.35^{b}	0.26^{b}	6.2^{c}
100-TVA	3.0^{c}	1.6^{c}	2.3^{a}	0.16^{c}	0.20^{b}	9.1^{a}
100-LA	4.7^{b}	2.7^{b}	1.3^{b}	0.29^{b}	0.21^{b}	6.1^{c}
100-CLA	3.8^{c}	1.3^{c}	2.5^{a}	0.18^{c}	0.17^{b}	8.0^{b}
Standard Error	0.18	0.02	0.01	0.02	0.02	0.08

*SA is stearic acid, OA is oleic acid, TVA is *trans*-vaccenic acid, LA is linoleic acid and CLA is conjugated linoleic acid.

[1]Different superscripts (a, b, c) indicate significant differences at $P < 0.05$ between fatty acid treatments within the same concentration.

[2]ACC activity expressed as nanomoles malonate formed from $NaH^{14}CO_3$ per mg cell protein.

[3]FAS activity expressed as nanomoles palmitate formed from 2-^{14}C-malonyl-CoA per mg cell protein.

[4]SCD activity expressed as nanomoles oleate formed from ^{14}C-stearoyl-CoA per mg cell protein.

[5]mRNA abundance expressed as ratio of density of the respective mRNA band to density of β-actin mRNA band.

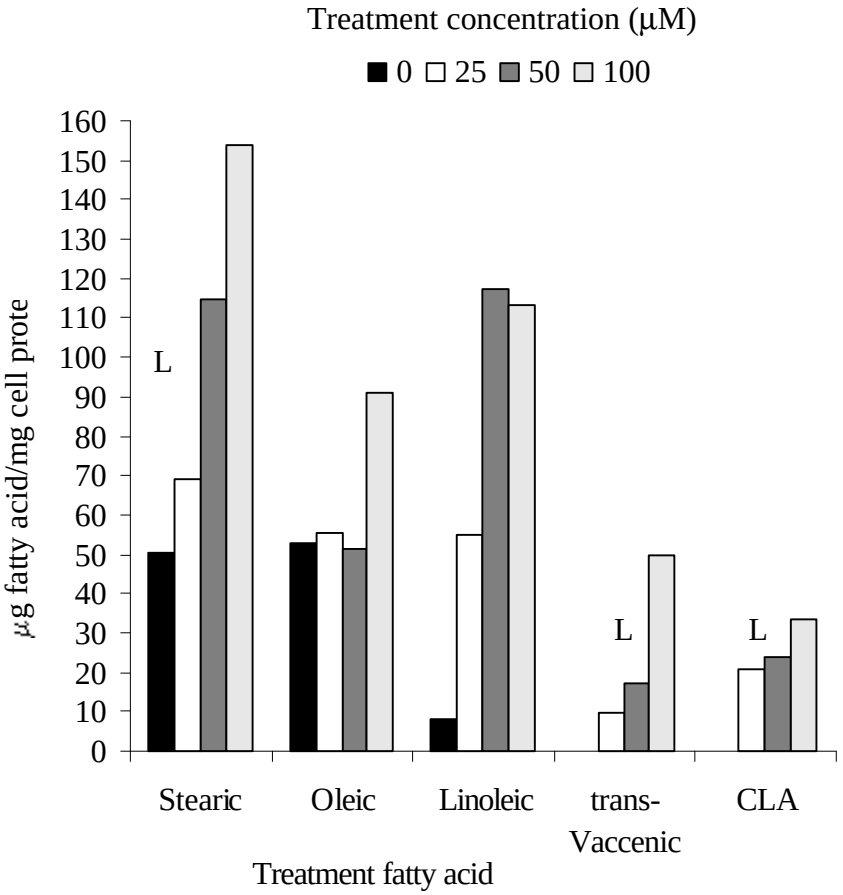

Figure 4.1
Treatment fatty acid concentration (µM) versus cellular fatty acid content (µg fatty acid per mg protein) in MacT cells. L indicates significant linear response

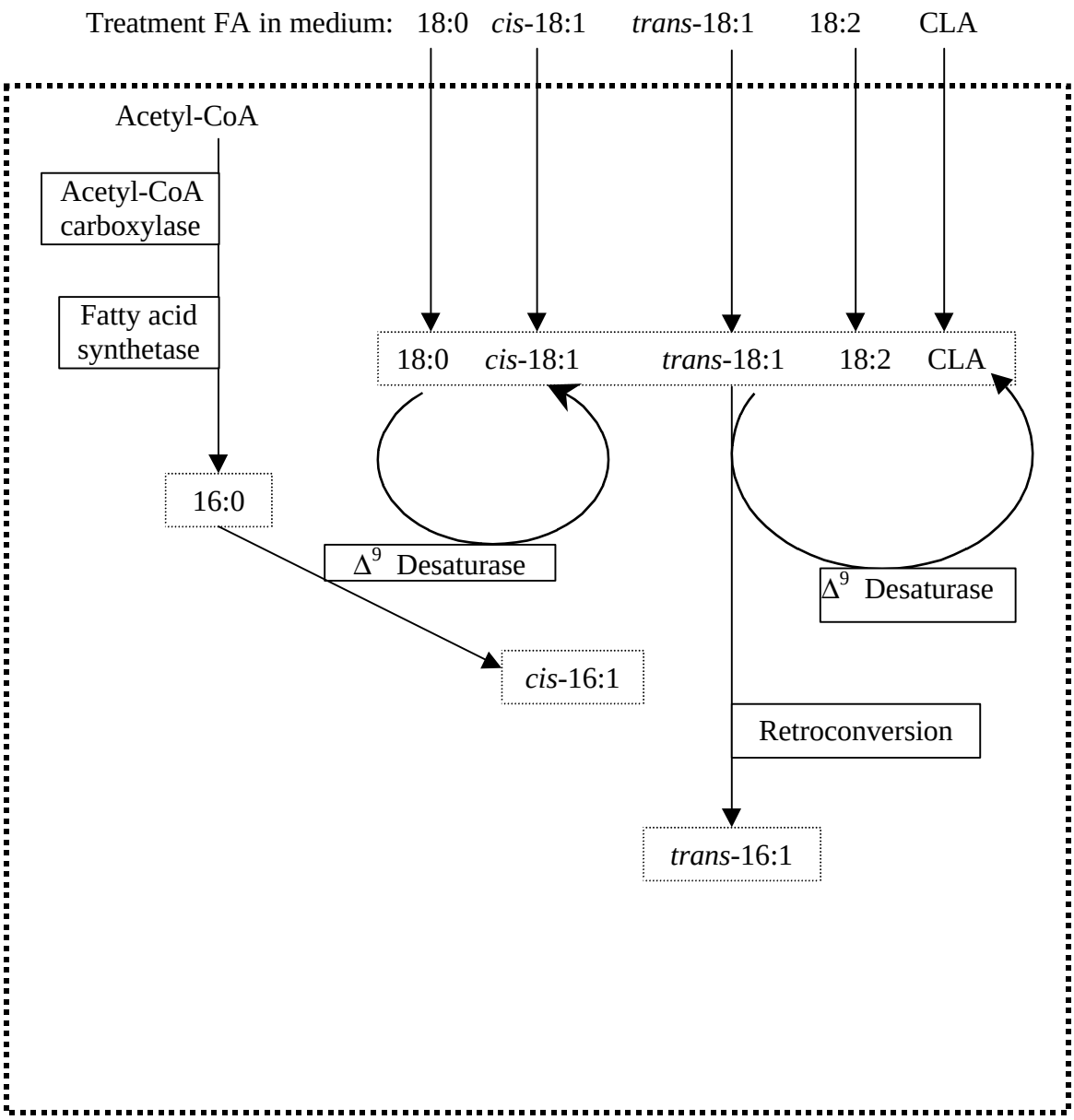

Figure 4.2
Interaction between treatment fatty acids (FA) and *de novo* FA synthesis in MacT cells.

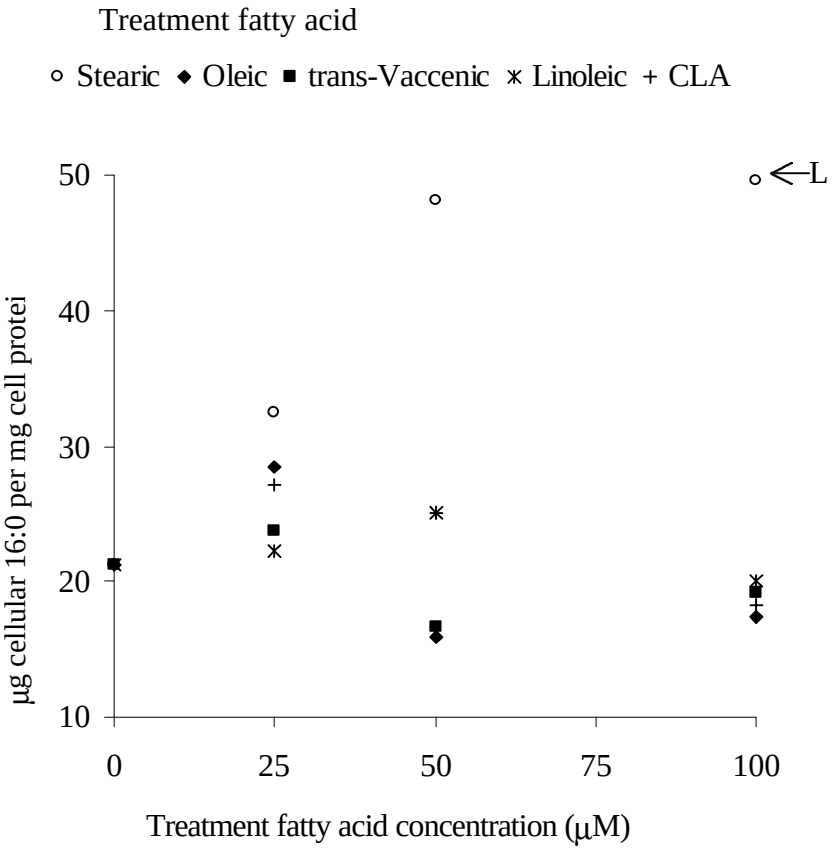

Figure 4.3
Cellular 16:0 (µg per mg protein) content in MacT cells in response to fatty acid treatment. L indicates significant linear response.

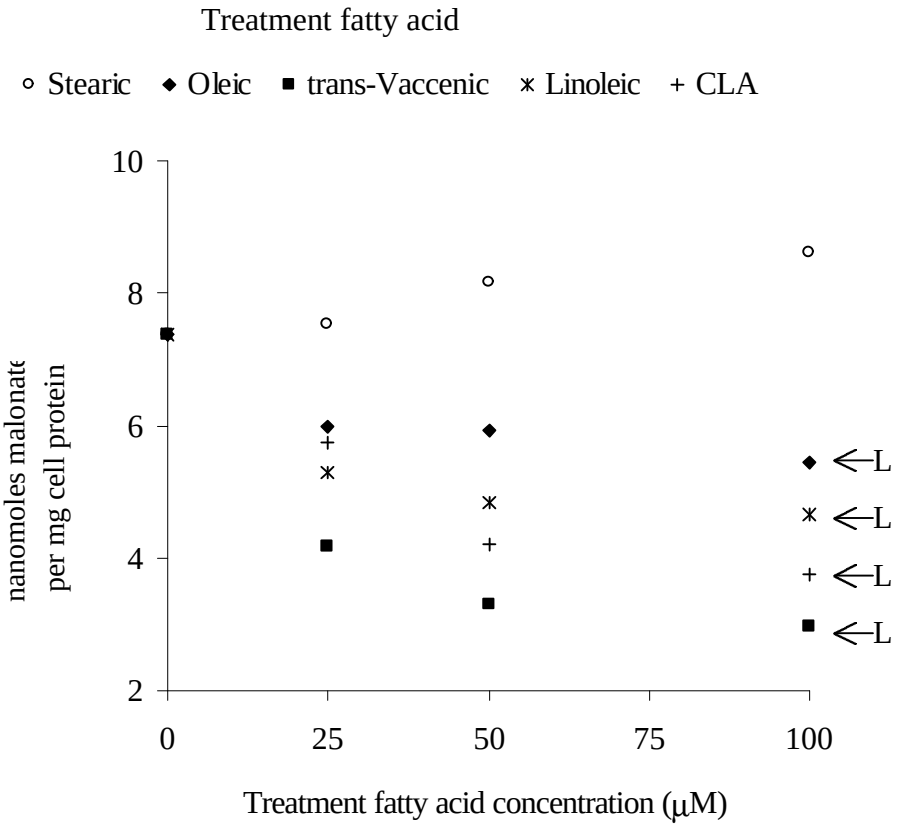

Figure 4.4
Cellular acetyl-CoA carboxylase (ACC) activity (expressed as nanomoles malonate formed from $NaH^{14}CO_3$ per mg protein) in MacT cells in response to fatty acid treatment. L indicates significant linear response.

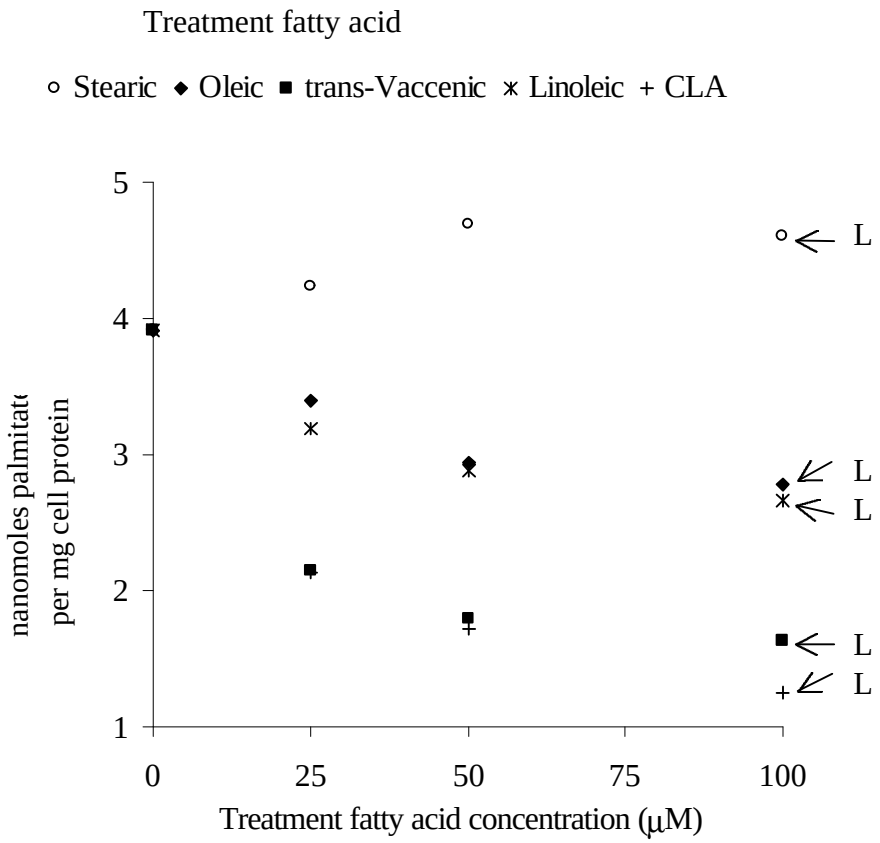

Figure 4.5
Cellular fatty acid synthetase (FAS) activity (expressed as nanomoles palmitate formed from 2-^{14}C-malonyl-CoA per mg protein) in MacT cells in response to fatty acid treatment. L indicates significant linear response.

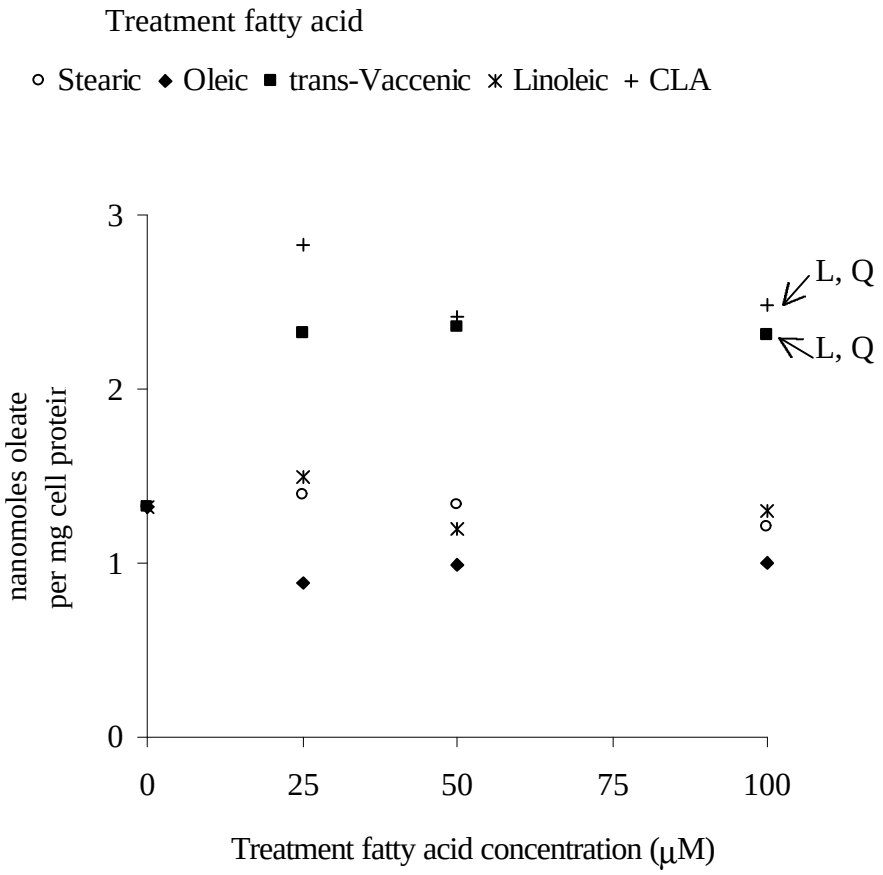

Figure 4.6
Cellular stearoyl-CoA desaturase (SCD) activity (expressed as nanomoles oleate formed from ^{14}C-stearoyl-CoA per mg protein) in MacT cells in response to fatty acid treatment. L indicates significant linear response and Q indicates significant quadratic response.

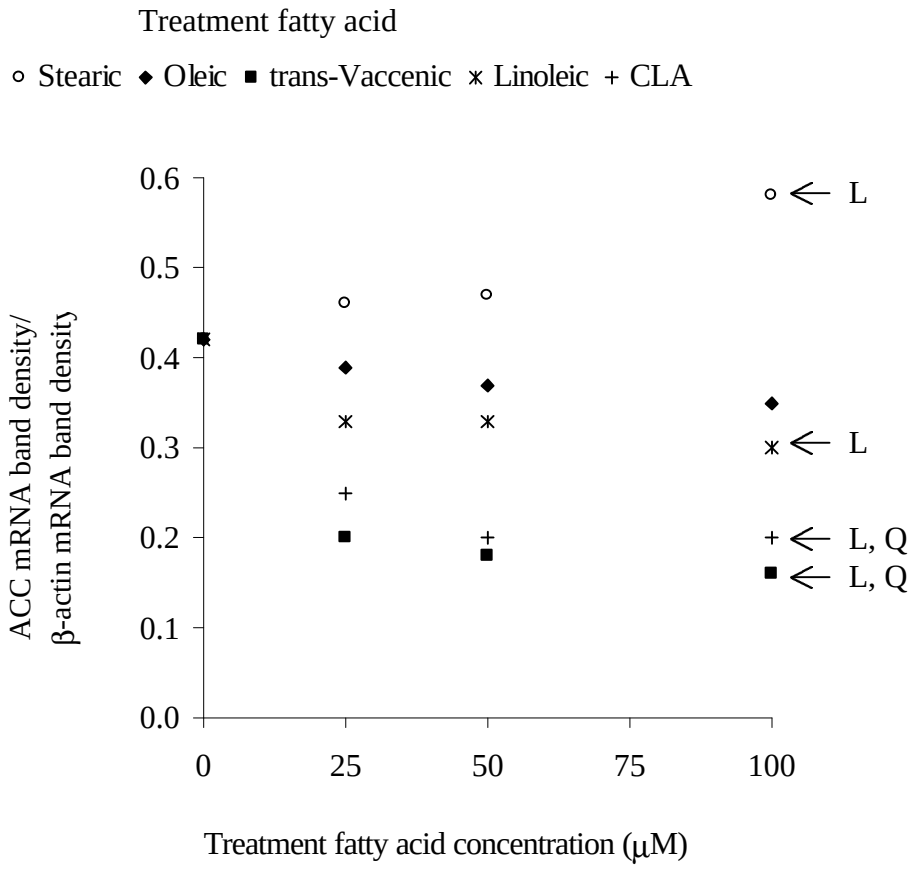

Figure 4.7
Cellular acetyl-CoA carboxylase (ACC) mRNA abundance (expressed as ratio of density of ACC mRNA band to density of β-actin mRNA band) in MacT cells in response to fatty acid treatment. L indicates significant linear response and Q indicates significant quadratic response.

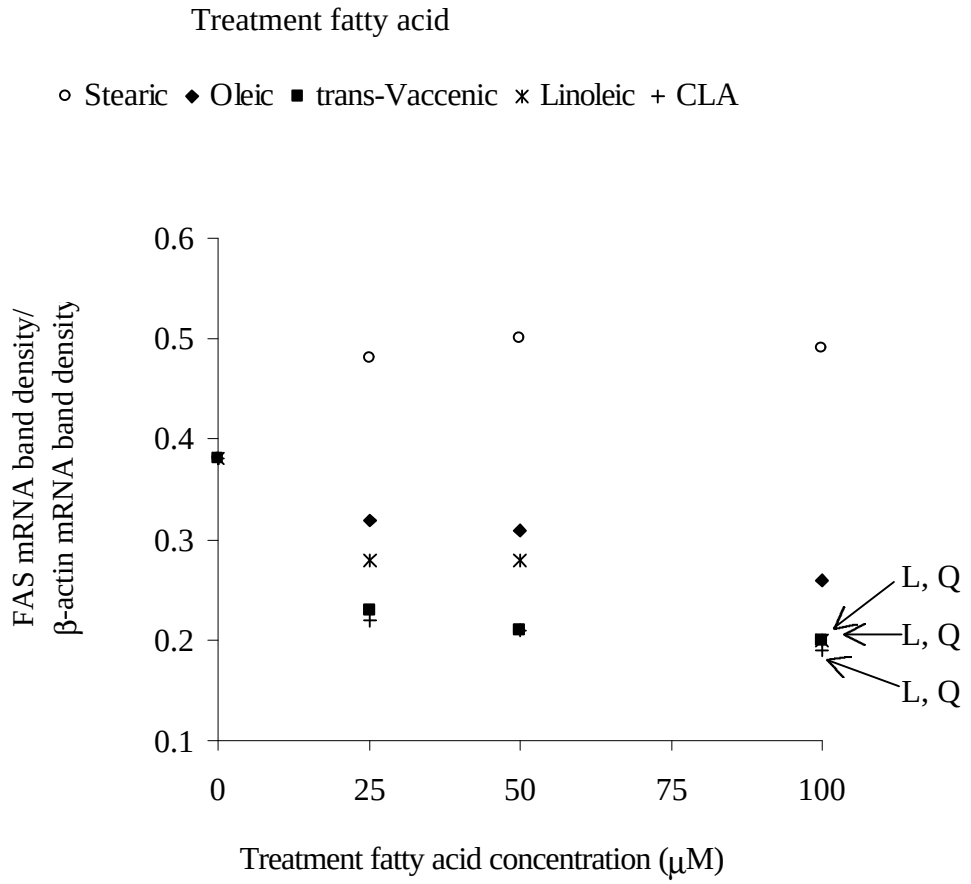

Figure 4.8
Cellular fatty acid synthetase (FAS) mRNA abundance (expressed as ratio of density of FAS mRNA band to density of β-actin mRNA band) in MacT cells in response to fatty acid treatment. L indicates significant linear response and Q indicates significant quadratic response.

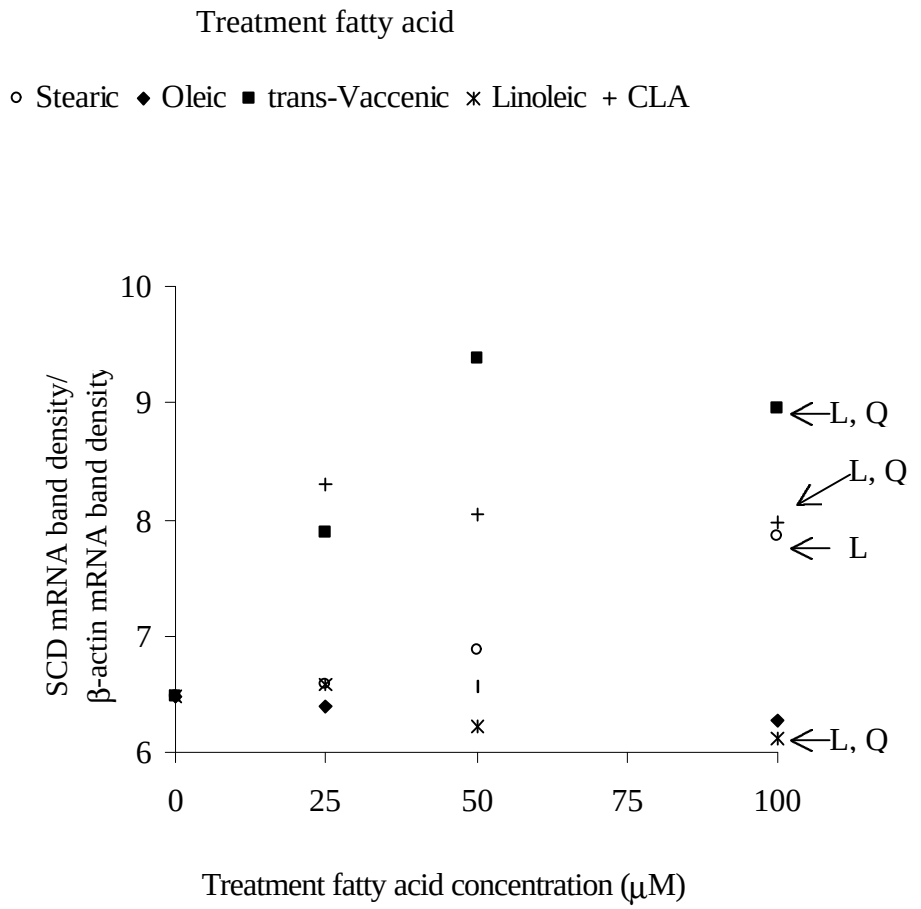

Figure 4.9
Cellular stearoyl-CoA desaturase (SCD) mRNA abundance (expressed as ratio of density of SCD mRNA band to density of β-actin mRNA band) in MacT cells in response to fatty acid treatment. L indicates significant linear response and Q indicates significant quadratic response.