CHAPTER 3

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

Abstract

Objectives of the study were to examine the effects of *cis* and *trans* isomers of 18:1 and 18:2 acids on fatty acid synthesis and desaturation in mouse mammary epithelial (MME) cells. The COMMA-D/MME cells, a subclone of the COMMA-D cell line, was used for the study. 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 was added daily to the cells for 3 days. The basal medium contained 100 μM stearic acid (18:0), to which 12.5, 25, 50 or 100μM of stearic acid, oleic acid, elaidic 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 MME cells. When compared with 18:0, all unsaturated fatty acids reduced cellular 16:0 content, which was indicative of 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 treatment-fatty acids inhibited cellular fatty acid synthetase activity. The extent of inhibition of fatty acid synthetase activity was greatest for conjugated linoleic acid, followed by oleic acid, *trans*-vaccenic acid, elaidic acid and linoleic acid.

Increasing concentrations of oleic and linoleic acids inhibited cellular desaturase activity and reduced desaturase mRNA abundance. Compared with stearic acid, the *trans*-vaccenic and elaidic acid treatments stimulated desaturase activity and mRNA abundance at concentrations of 12.5 to 50 μM. However, both *trans*-18:1 isomers caused concentration-dependent inhibition of cellular desaturase activity and desaturase mRNA abundance. Data indicated depression of *de novo* fatty acid synthesis and desaturation in the mammary gland by *cis* isomers of 18:1 and 18:2. *Trans*-18:1 isomers inhibited mammary synthesis of saturated fatty acids but enhanced Δ^9 desaturation at low concentrations.

Introduction

Typical milk fat from a dairy cow contains approximately 5% polyunsaturated fatty acids (PUFA), 70% saturated fatty acids (SFA), and 25% monounsaturated fatty acids (MUFA) (Grummer, 1991). Due to strong evidence for a positive correlation between dietary SFA intake and incidence of coronary heart disease, there is increasing demand to lower the SFA content of dairy products. The 1988 Wisconsin Milk Marketing Board's Milk Fat Roundtable concluded that the "ideal" nutritional milk fat would contain up to 10% PUFA, less than 8% SFA, and greater than 82% MUFA (O'Donnell, 1989). This large discrepancy between the "ideal" and actual cannot be easily resolved through dietary manipulation. However, supplementing the diet of a dairy cow with certain unsaturated fatty acids can improve the ratio of unsaturated to saturated fatty acids in milk fat.

A recent study conducted in our lab used abomasal infusion of a mixture of linoleic and conjugated linoleic acids (100g each, over a 24 hour period) to Holstein cows. Results indicated a simultaneous increase in the unsaturated fatty acid content of milk fat from 23 to 45%, and a decrease in the SFA content from 70 to 42% (Loor and Herbein, 1997). Another study (Loor et al., 1998) in which soybean oil was fed to Jersey cows indicated that soybean oil intake increased the levels of linoleic, conjugated linoleic and *trans*-vaccenic acids in blood plasma. It was also seen that the levels of linoleic, conjugated linoleic and *trans*-vaccenic acids in milk fat were elevated during soy-oil feeding, and there was a linear relationship between their concentrations in blood plasma and milk. Being a PUFA, increasing the concentration of conjugated linoleic acid in milk fat would

certainly be beneficial. Moreover, CLA has been reported to have potential anti-carcinogenic properties (Parodi, 1994).

Even though studies have indicated that dietary unsaturated fatty acids altered milk fat content and composition, the factors responsible for changes in milk fat composition in response to dietary fatty acids are not known. Studies at the cellular level in mammary cells are necessary to understand the influence of external fatty acids on cellular factors (such as lipogenic enzymes) involved in milk fat synthesis. The present study used mouse mammary epithelial cells to determine the effect of external *cis* and *trans* isomers of 18:1 and 18:2 on mammary fatty acid synthesis and desaturation.

Materials and Methods

Cell culture

Mouse mammary epithelial (MME) cells, COMMA-D/MME, a subclone of the COMMA-D cell line 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 and pH was adjusted to 7.4. The medium was also supplemented with 10 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 a 0.2 μ surfactant free cellulose acetate (SFCA) filter (Nalgene, NY).

Treatment media were supplemented with treatment fatty acids (FA). The treatments are shown in Table 3.1. 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 and cells were rinsed with phosphate buffered saline (PBS). The cells were then covered with sucrose buffer for 15 min at room temperature. After 15 min, the sucrose buffer was removed and cells were covered with trypsin/EDTA (0.5%/0.2%) (Sigma) in Hank's balanced salt solution without calcium chloride, magnesium sulfate or sodium bicarbonate (Sigma), and incubated at 37^{o}C for 20 min. At the end of 20 min, trypsin activity was inhibited by adding medium containing FBS. The mixture was then transferred to a sterile plastic centrifuge tube and the cells were pelleted by centrifuging 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. The sucrose buffer contained 100 mM Na_2HPO_4, 10 mM EDTA, 28.2 mM sucrose, and 1 g/L bovine serum albumin.

A total of 20 plates were used for each treatment. Four 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.

Four sets of one plate each were taken for estimation of protein and DNA contents and stearoyl-CoA desaturase (SCD) and fatty acid synthetase (FAS) activities. Each plate

was resuspended in 1 mL PBS. Aliquots of 250 μL were collected into microfuge tubes and stored at -70^{o}C until analysis.

Four plates were taken for extraction of total RNA and Northern blot analysis of SCD mRNA abundance. Total RNA was immediately extracted, 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.

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 in terms of the amount of oleate formed per mg cell protein.

Extraction of total RNA

Total RNA from the cells was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). The harvested cells were lysed using 4 M guanidinium thiocyanate containing 25 mM sodium citrate pH 7, 0.5% sarkosyl and 0.1M mercaptoethanol in DEPC-treated water. DEPC is diethyl pyrocarbonate. The RNA from the cell lysate was extracted with a mixture containing 2M sodium citrate, phenol and chloroform in the ratio 1:10:2 (v/v/v). The extracted RNA was then precipitated with isopropanol, washed in 70% ethanol and solubilized in 10 mM Tris-HCl pH 7.5 containing 1mM EDTA and 0.5% SDS. (EDTA is ethylene diamine tetra acetic acid, and SDS is sodium dodecyl sulfate). From this solution, the RNA was again extracted using a mixture of chloroform and 3 M sodium acetate (10:1, v/v), then precipitated with isopropanol and washed in 70% ethanol. Finally, the RNA was 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 SCD mRNA abundance

Total RNA (15 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 is 10X saturated sodium citrate and 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 SCD mRNA. Concentration of probe in the hybridization solution was 10 ng/mL. The prehybridization/hybridization solution contained 50% formamide, 5X SSPE pH 7.4, 5X Denhardt's solution, 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. 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 in 5X SSPE-0.5% SDS at room temperature, for 15 min each, then twice for 15 min each in 1X SSPE-0.5% SDS at 37^0C, and finally in 0.1X SSPE-1% SDS for 15 min at 37^oC). Subsequently, binding of the labeled probe to target

mRNA was visualized by autoradiography of the hybridized membrane on 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 desaturase mRNA bands, it was attempted to rehybridize the membrane with a labeled β-actin probe, after stripping it with 0.1X SSC containing 1% SDS. When this attempt failed, an attempt was made to rehybridize the membrane to an 18S rRNA probe. But, since the membrane did not hybridize to either of these probes, the SCD mRNA was quantitated by scanning the 18S rRNA band and the SCD mRNA band on polaroid pictures of the electrophoresis gel. The SCD mRNA abundance was expressed as the ratio of the desaturase mRNA band to that of 18S rRNA band.

The cDNA probe for SCD mRNA was kindly donated by Dr. James Ntambi, University of Wisconsin, Madison. Sufficient quantities of the probe were prepared (Appendix I) for use in Northern blot analysis.

Statistical analysis

Data for cellular protein, DNA and FA contents 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 the 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 $P < 0.05$.

Data for FAS and SCD activities and SCD mRNA abundance were not statistically analyzed, because there were no replicates for the determination. Hence these data are presented in Table 3.4 without indicating the standard error, and in Figures 3.5, 3.6 and 3.7 without indicating the nature of cellular response.

Results and Discussion

Cell protein and DNA contents

Protein and DNA contents in MME cells treated with the different fatty acids (FA) are given in Table 3.2. In general, cellular protein and DNA contents are indicators of cell number and cell size. But in the present study the protein and DNA contents were measured in order to express the other cellular parameters on protein or DNA basis. However, it was interesting to note that all treatment concentrations of conjugated linoleic acid (CLA) significantly reduced the amounts of cellular DNA and protein per plate, when compared with other treatments. This is indicative of reduction in the number of cells per plate. The MME cells used in the experiment was a mouse mammary cell line that was immortalized with a bovine papilloma virus gene (Gibson and Baumrucker, 1996). CLA has been reported to have potential anticarcinogenic activity, and inhibited proliferation of different varieties of tumor cells (Cornell et al., 1997). So, the reduction in cell number caused by CLA could be due to the antiproliferative activity of CLA towards the 'immortal' gene in MME cells.

Cellular FA profile

The FA content in MME cells is given in Table 3.3. There was a positive relationship between media concentration of a treatment FA and its concentration in the cells. This indicates 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 3.1.

Cellular 16:0 content

The media did not contain 16:0, but acetate which is the precursor for 16:0 synthesis was provided in the media. So, the most likely source of 16:0 in the MME 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 content in response to FA treatment is shown in Figures 3.2 and 3.3.

Stearic acid (SA) treatment caused a concentration-dependent increase in cellular 16:0 content. This indicates that SA enhanced *de novo* FA synthesis by the cells. At all concentrations, CLA significantly reduced cellular 16:0 content when compared with the other treatments, suggesting CLA inhibited *de novo* FA synthesis. In an early study, Dawson and Herbein (1996) observed that external CLA reduced cellular 16:0 content in MME cells in a concentration-dependent manner. The results indicate that CLA was potentially inhibiting cellular FAS activity. It is also evident from Figure 3.3 that increasing concentrations of elaidic acid (EA) or *trans*-vaccenic acid (TVA) had very little effect on the 16:0 content in MME cells. At 12.5 to 50 μM, oleic acid (OA) and linoleic acid (LA) did not significantly alter cellular 16:0 content, when compared with SA (Table 3.3). However, at 100 μM OA and LA significantly reduced cellular 16:0 when compared with SA.

Cellular 18:0 content

Cellular 18:0 content in MME cells increased with increasing concentration of SA in the media (Table 3.3). Cellular 18:0 content also increased from the control level as the concentration of LA and OA in the treatment media increased. This could be due to 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 will increase their fluidity and may disrupt normal physiological status. In order to compensate for this disruption, it is reasonable that the cells would take up more of the 18:0 available from the media. Cellular 18:0 content also increased with increase in the amount of CLA in the media. This is also probably due to enhanced uptake of 18:0 to maintain normal membrane fluidity.

Increasing concentrations of EA or TVA had very little effect on cellular 18:0 content. This could be because EA and TVA are *trans*-18:1 isomers, and hence behave more like saturated FA in their physical properties. Consequently, greater incorporation of these FA into the cell membrane is not likely to disrupt membrane fluidity as much as their *cis* counterparts. So, there is no need for the cells to take up more of 18:0 to maintain normal membrane fluidity.

Cellular *cis*18:1 content

SA treatment produced a concentration-dependent linear increase in cellular *cis*-18:1 content (Figure 3.4). SA was taken up by the cells in a concentration-dependent manner (Table 3.3). Consequently, there is likely to be increased incorporation of this saturated FA into the cell membranes, leading to reduced membrane fluidity. As a response to maintain normal membrane fluidity, the cells would attempt to increase their unsaturated FA content by desaturating the available 18:0. The primary product of 18:0 desaturation is *cis*-18:1. So, it is possible that SA treatment increased cellular desaturase activity.

EA, TVA, LA and CLA caused a linear concentration-dependent decrease in cellular *cis*-18:1 content (Figure 3.4). This is most likely to be due to reduced cellular desaturase activity in response to the treatments.

Cellular *cis*-16:1 content

The most likely sources of *cis*-16:1 in the cells are desaturation of the *de novo* synthesized16:0, or retroconversion of *cis*-18:1. Increasing concentrations of OA and LA produced a concentration-dependent decrease in cellular *cis*-16:1 content (Table 3.3). This was probably due to inhibition of desaturation of 16:0, and/or due to reduced availability of 16:0 for desaturation. At all concentrations, CLA reduced cellular *cis*-16:1, when compared with SA. At 12.5 to 50 μM, EA and TVA did not significantly alter cellular *cis*-16:1 when compared with SA. However, at 100 μM EA and TVA significantly lowered *cis*-16:1 when compared with SA, and this could be due to

inhibition of cellular desaturase activity or due to lesser amount of 16:0 available for desaturation.

Cellular *trans*-16:1 content

From Table 3.3 it can be seen that only the cells treated with EA or TVA contained detectable amounts of *trans*-16:1. There was a significant positive linear relationship between cellular *trans*-16:1 and the concentration of EA or TVA in the treatment media. At all treatment concentrations, the content was higher in the EA-treated cells when compared with the TVA-treated cells. It is also important to note that the concentration of cellular EA in the EA-treated cells was higher than the concentration of TVA in the TVA-treated cells, and this was probably because EA was more readily taken up by the cells when compared with TVA.

Neither *cis*- nor *trans*-16:1 was supplemented in the media, and the product of 16:0 desaturation is *cis*-16:1. So, the only possible source of *trans*-16:1 in the cells would be incomplete peroxisomal β-oxidation of the *trans*-18:1 isomers. Retroconversion of *trans*-monoene fatty acids have been reported previously. Bourre et al. (1982) observed retroconversion of EA 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. In the present study, the higher amount of *trans*-16:1 in the EA-treated cells when compared with the TVA-treated cells corresponds to the greater uptake of EA by the cells. This also supports the suggestion that the *trans*-16:1 in MME cells was from retroconversion of *trans*-18:1 isomers.

Cellular FAS activity

FAS activity in MME cells in response to FA treatment is shown in Figure 3.5. Treatment with SA enhanced cellular FAS activity in a concentration-dependent manner. OA, EA, TVA, LA and CLA inhibited activity of cellular FAS in a concentration-dependent manner, with CLA being the most potent inhibitor.

FAS is one of the lipogenic enzymes that has been positively related to the rate of *de novo* FA synthesis in tissues. The FAS enzyme basically catalyzes the synthesis of long-chain FA, primarily 16:0, from acetyl-CoA and malonyl-CoA. In the present study, increasing concentrations of SA also caused a concentration-dependent increase in cellular 16:0 content (Table 3.3). This increase in 16:0 content could have been a result of increased FAS activity, which enhanced the rate of conversion of acetate to 16:0.

There has been several reports suggesting that dietary unsaturated FA were potential inhibitors and that saturated FA were enhancers of *de novo* FA synthesis in various tissues. Strum-Odin et al. (1987) found that FAS activity increased in hepatocytes conditioned to media containing 16:0. 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. Park et al. (1997) reported that dietary CLA reduced body fat deposition and increased lipolysis in adipocytes of mice. However, the mechanism of depression of fatty acid synthesis observed in the above studies was not elucidated. Results of the present

study suggest that those effects were due to inhibition of cellular FAS activity by *cis* and *trans* isomers of 18:1 and 18:2.

However, more investigation is necessary to understand the processes involved in inhibition of FAS activity. Enhanced FAS activity could result from the presence of increased amounts of the enzyme consequent to enhanced transcription from the gene, or due to greater stimulation of the activity of already existing enzyme. Likewise, inhibition of *de novo* synthesis could result from reduced rate of transcription or due to lower stimulation of the enzyme molecules. Paulauskis and Sul (1989) 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. It has also been shown that the effect of dietary factors on FAS activity was tissue-specific (Kim and Freake, 1996).

Cellular SCD activity

SCD activity in MME cells in response to FA treatment is shown in Figure 3.6. SA resulted in a concentration-dependent increase in cellular SCD activity. OA caused a steady decrease in SCD activity with increasing concentrations. In the case of EA, TVA, LA and CLA, the enzyme activity was higher than that of the control, but with increasing treatment concentration there was an eventual decline in activity from the control level.

In eukaryotes, saturated FA are precursors of unsaturated FA. Introduction of the first *cis*-double bond at the Δ^9 position is a critical step in the synthesis of unsaturated FA

from saturated FA, and the reaction is catalyzed by the Δ^9 desaturase system. SCD is capable of catalyzing Δ^9 desaturation of various methylene-interrupted fatty acyl-CoA substrates, but palmitoyl- and stearoyl-CoA are the preferred substrates (Ntambi, 1995). These substrates are converted to 16:1 and 18:1, respectively. Studies have also indicated that response of desaturase activity to dietary and hormonal factors was tissue-specific. Enser (1975) found that SCD activity in the liver and adipose tissue of obese mice were significantly higher than that of their lean counterparts. However, increased activity in the liver of obese mice was due to increased enzyme activity per cell, whereas in the adipose tissue the increased activity was a result of tissue hyperplasia, and not due to increased activity per cell.

Most studies on SCD activity have focused on the liver and adipose tissue or the corresponding cell cultures, and there has been very few reports on the influence of dietary factors on SCD activity in mammary cells. In the present study, it was seen that in addition to enhancing cellular SCD activity (Figure 3.6) SA-treatment also increased 18:1 concentration in the cells in a concentration-dependent manner, and that SA was taken up by the MME cells in a concentration-dependent manner (Table 3.3). So, the enhanced SCD activity in response to SA could be a physiological response to maintain normal membrane fluidity. Likewise, the decrease in SCD activity due to LA could be a physiological response to maintain a balance between saturated and unsaturated FA in cell membranes. Inhibition of SCD activity by OA could be due to product-inhibition of the enzyme, or it may also be a cellular response to maintain a balance between saturated and unsaturated FA in the cell membranes.

EA and TVA are *trans*-18:1 isomers, and they have physical properties that are in between the corresponding saturated and *cis* isomers (Ovesen and Leth, 1995). So, their incorporation into cell membranes would not disrupt normal membrane fluidity as much as their *cis* counterparts. However, as treatment-concentration of EA or TVA increased, there was a gradual decline in SCD activity. This was probably due to disruption of normal membrane fluidity as more amounts of the *trans* isomer incorporated into the cell membrane.

Cellular SCD mRNA abundance

Effect of FA on SCD mRNA abundance followed a similar pattern as for SCD enzyme activity (Figure 3.7). SA produced a concentration-dependent increase in SCD mRNA abundance, whereas OA, LA and CLA reduced SCD mRNA abundance. The inhibitory effect on mRNA abundance by CLA was evident only at 50 and 100 μM, and the effect by LA was evident only 25, 50 and 100 μM. EA and TVA increased the abundance of desaturase mRNA when compared with the control, but with increasing treatment concentrations there was a gradual decrease in the amount of desaturase mRNA. These observations suggested that the treatments not only affected activity of desaturase enzyme, but they were capable of modulating transcription of the desaturase gene. Similar results have been reported in previous studies using tissues or cells other than the mammary gland. Paisley et al. (1996) reported that dietary corn oil reduced the abundance of SCD 1 mRNA in mouse liver. Sessler et al. (1996) found that arachidonic acid, linoleic acid and linolenic acid decreased SCD 1 mRNA abundance in mature adipocyte cultures by reducing the half-life of the mRNA.

Abundance of an mRNA is an indicator of its rate of synthesis (transcription) and degradation. However, the differences in mRNA abundance due to treatments could be due to direct control of the 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 desaturase protein were not measured. So, it is not possible to conclude whether the treatment FA influenced SCD gene transcription or not. Further research is needed to establish the cause of altered mRNA abundance in response to FA treatment.

Table 3.1
Concentration of supplemental fatty acids in treatment media applied to confluent COMMA-D/MME cell cultures

	Supplemental fatty acid concentration in treatment media (μM)					
Treatment*	SA*	OA*	EA*	TVA*	LA*	CLA*
Control	100					
12.5-SA	112.5					
25-SA	125					
50-SA	150					
100-SA	200					
12.5-OA	100	12.5				
25-OA	100	25				
50-OA	100	50				
100-OA	100	100				
12.5-EA	100		12.5			
25-EA	100		25			
50-EA	100		50			
100-EA	100		100			
12.5-TVA	100			12.5		
25-TVA	100			25		
50-TVA	100			50		
100-TVA	100			100		
12.5-LA	100				12.5	
25-LA	100				25	
50-LA	100				50	
100-LA	100				100	
12.5-CLA	100					12.5
25-CLA	100					25
50-CLA	100					50
100-CLA	100					100

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

Table 3.2
Cellular protein and DNA contents in COMMA-D/MME cells in response to fatty acid supplementation.

Treatment*	Cell fraction (mg per plate) [1]	
	Protein	DNA
Control	4.01	0.80
12.5-SA	4.22[a]	0.90[a]
12.5-OA	4.11[a]	0.80[b]
12.5-EA	4.01[a]	0.81[b]
12.5-TVA	4.03[a]	0.80[b]
12.5-LA	4.02[a]	0.80[b]
12.5-CLA	3.51[b]	0.71[c]
25-SA	4.11[a]	0.90[b]
25-OA	4.16[a]	1.00[a]
25-EA	4.16[a]	0.81[c]
25-TVA	4.04[a]	0.80[c]
25-LA	4.03[a]	0.81[c]
25-CLA	3.01[b]	0.72[d]
50-SA	4.21[a]	0.91[ab]
50-OA	4.14[a]	1.01[a]
50-EA	4.13[a]	0.81[c]
50-TVA	3.92[a]	0.80[c]
50-LA	4.02[a]	0.90[b]
50-CLA	2.72[b]	0.61[d]
100-SA	3.11[b]	0.71[c]
100-OA	4.40[a]	1.01[a]
100-EA	3.40[b]	0.80[b]
100-TVA	4.11[a]	0.71[c]
100-LA	4.02[a]	0.80[b]
100-CLA	2.22[c]	0.60[d]
Standard Error	0.09	0.01

*SA is stearic acid, OA is oleic acid, EA is elaidic acid, TVA is *trans*-vaccenic acid, LA

is linoleic acid and CLA is conjugated linoleic acid.

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

Table 3.3
Cellular fatty acid profile in COMMA-D/MME cells in response to fatty acid treatment

Treatment*	Cellular content (μg per mg protein)[1]								
	16:0	*trans* 16:1	*cis* 16:1	18:0	*trans* 18:1 (Δ9)	*trans* 18:1 (Δ11)	*cis* 18:1	18:2	CLA
Control	16.8	0	4.0	7.9	0.8	0	31.6	0.5	0
12.5-SA	18.5^{ab}	0^{c}	4.5^{a}	10.0^{ab}	0.7^{bc}	0^{b}	35.8^{ab}	0.2^{b}	0.3^{b}
12.5-OA	20.8^{ab}	0^{c}	4.3^{a}	9.5^{ab}	1.3^{abc}	0^{b}	39.2^{a}	0.9^{b}	1.8^{a}
12.5-EA	16.3^{ab}	1.7^{a}	4.5^{a}	7.2^{b}	2.6^{a}	0^{b}	30.0^{bc}	1.0^{b}	1.2^{a}
12.5-TVA	15.0^{b}	0.5^{b}	3.7^{b}	6.5^{c}	0^{c}	2.4^{a}	24.7^{c}	1.2^{b}	1.2^{a}
12.5-LA	19.1^{ab}	0^{c}	2.8^{b}	11.4^{a}	1.7^{ab}	0^{b}	27.0^{c}	8.8^{a}	1.3^{a}
12.5-CLA	9.3^{c}	0^{c}	3.1^{b}	6.6^{c}	1.1^{abc}	0^{b}	24.9^{c}	0.6^{b}	1.0^{a}
25-SA	19.4^{a}	0^{c}	4.4^{a}	10.3^{a}	1.8^{b}	0^{b}	37.3^{a}	1.1^{b}	0.2^{c}
25-OA	19.2^{a}	0^{c}	3.4^{c}	8.6^{bc}	1.2^{b}	0^{b}	34.9^{a}	0.7^{b}	1.6^{b}
25-EA	16.2^{a}	3.0^{a}	4.1^{ab}	7.3^{c}	4.8^{a}	0^{b}	27.3^{b}	0.7^{b}	1.1^{b}
25-TVA	16.0^{a}	1.2^{b}	4.3^{a}	6.7^{d}	0^{b}	2.6^{a}	27.3^{b}	0.4^{b}	1.5^{b}
25-LA	19.2^{a}	0^{c}	2.4^{c}	10.9^{a}	1.8^{b}	0^{b}	20.9^{c}	11.6^{a}	0.9^{c}
25-CLA	11.5^{b}	0^{c}	3.6^{bc}	9.3^{ab}	2.4^{b}	0^{b}	25.5^{b}	1.0^{b}	2.4^{a}
50-SA	20.0^{ab}	0^{c}	4.3^{a}	11.1^{a}	1.9^{bc}	0^{b}	40.1^{a}	1.4^{b}	0.1^{d}
50-OA	22.3^{a}	0^{c}	3.2^{b}	9.4^{b}	0.8^{cd}	0^{b}	42.8^{a}	0.4^{b}	2.2^{b}
50-EA	16.4^{b}	5.1^{a}	4.3^{a}	7.2^{c}	8.5^{a}	0^{b}	26.2^{b}	0.4^{b}	0.9^{c}
50-TVA	17.8^{ab}	2.5^{b}	4.6^{a}	7.2^{c}	0^{d}	5.7^{a}	27.3^{b}	0.6^{b}	2.1^{b}
50-LA	21.0^{a}	0^{c}	2.1^{c}	11.2^{a}	0.7^{cd}	0^{b}	15.9^{c}	16.7^{a}	0.7^{c}
50-CLA	10.7^{c}	0^{c}	4.0^{a}	9.2^{b}	2.7^{b}	0^{b}	24.0^{b}	1.9^{b}	6.9^{a}
100-SA	25.5^{a}	0^{c}	5.1^{a}	18.6^{a}	1.8^{bc}	0^{b}	56.0^{a}	0.8^{b}	0^{d}
100-OA	16.9^{b}	0^{c}	1.8^{c}	8.4^{c}	1.0^{c}	0^{b}	40.6^{b}	0.4^{b}	2.2^{b}
100-EA	16.7^{b}	9.3^{a}	3.7^{b}	7.5^{c}	18.1^{a}	0^{b}	24.8^{c}	0.5^{b}	0.9^{c}
100-TVA	17.1^{b}	5.2^{b}	3.8^{b}	6.6^{c}	0^{c}	9.4^{a}	23.2^{c}	0.5^{b}	2.8^{b}
100-LA	17.4^{b}	0^{c}	1.3^{c}	10.3^{b}	0.8^{c}	0^{b}	9.6^{d}	19.9^{a}	0.4^{c}
100-CLA	13.8^{b}	0^{c}	3.1^{b}	13.0^{b}	2.9^{b}	0^{b}	19.2^{c}	1.7^{b}	13.1^{a}
Standard Error	0.89	0.07	0.18	0.56	0.42	0.17	1.21	0.37	0.16

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

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

Table 3.4
Cellular fatty acid synthetase (FAS) and stearoyl-CoA desaturase (SCD) activities and SCD mRNA abundance in COMMA-D/MME cells in response to fatty acid treatment

Treatment*	FAS activity[1]	SCD activity[2]	SCD mRNA abundance[3]
Control	1.6	1.5	1.0
12.5-SA	1.7	1.5	1.1
12.5-OA	1.6	1.5	0.9
12.5-EA	1.4	3.2	1.3
12.5-TVA	1.3	3.8	1.3
12.5-LA	1.6	1.9	1.0
12.5-CLA	1.2	2.6	1.0
25-SA	1.8	1.6	1.1
25-OA	1.5	1.4	0.8
25-EA	1.4	2.7	1.2
25-TVA	1.3	3.3	1.3
25-LA	1.5	1.9	0.9
25-CLA	1.1	2.3	1.0
50-SA	1.8	1.8	1.2
50-OA	1.4	1.4	0.6
50-EA	1.3	2.3	1.2
50-TVA	1.3	2.9	1.2
50-LA	1.5	1.7	0.7
50-CLA	1.0	1.5	0.6
100-SA	2.6	3.3	1.3
100-OA	1.3	1.2	0.5
100-EA	1.2	2.3	1.2
100-TVA	1.1	2.6	1.2
100-LA	1.4	1.2	0.5
100-CLA	0.9	1.4	0.5

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

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

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

[3]SCD mRNA abundance expressed as ratio of density of SCD mRNA band to density of 18S rRNA band.

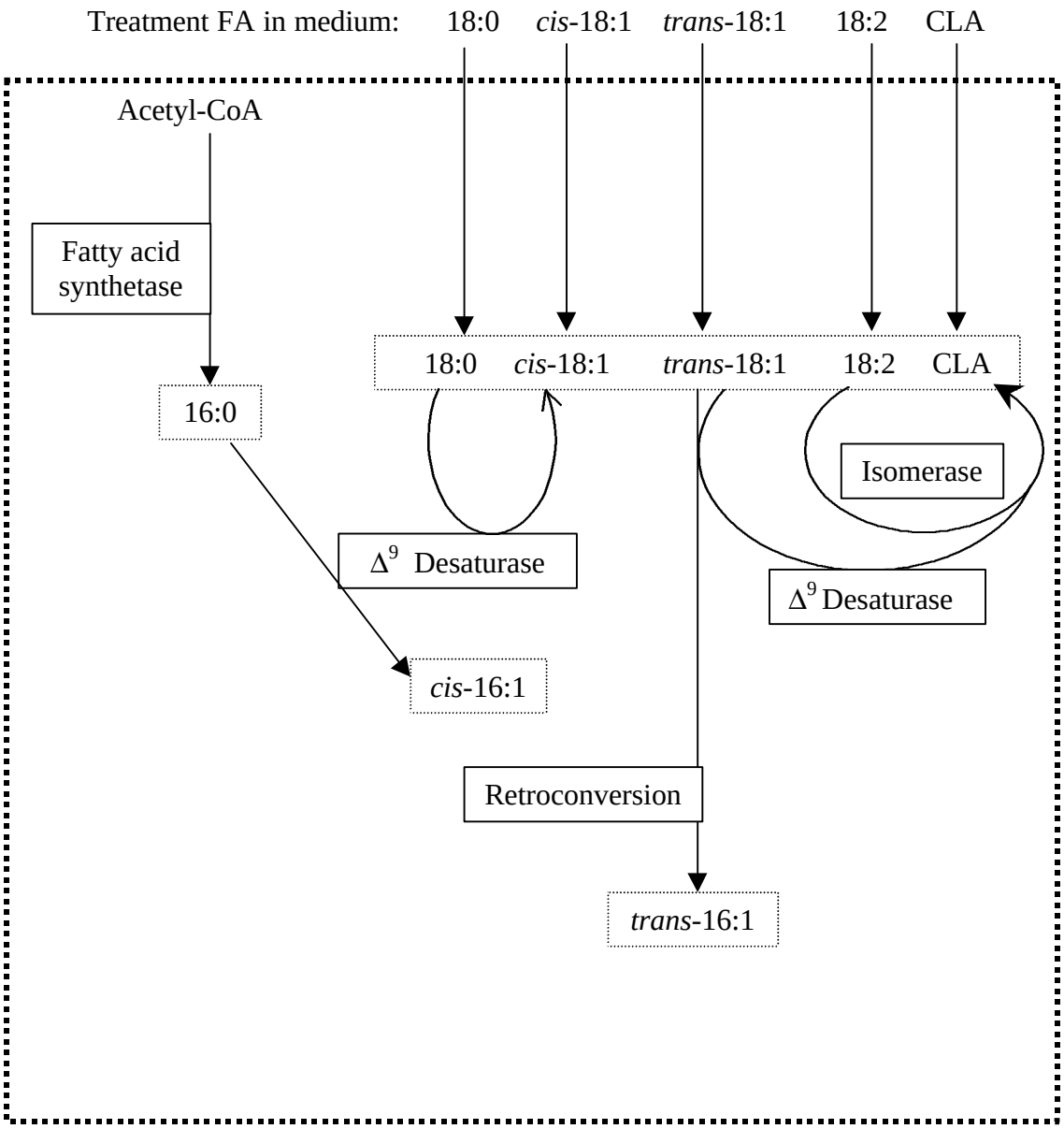

Figure 3.1
Interaction between treatment fatty acids (FA) and *de novo* FA synthesis in COMMA-D/MME cells

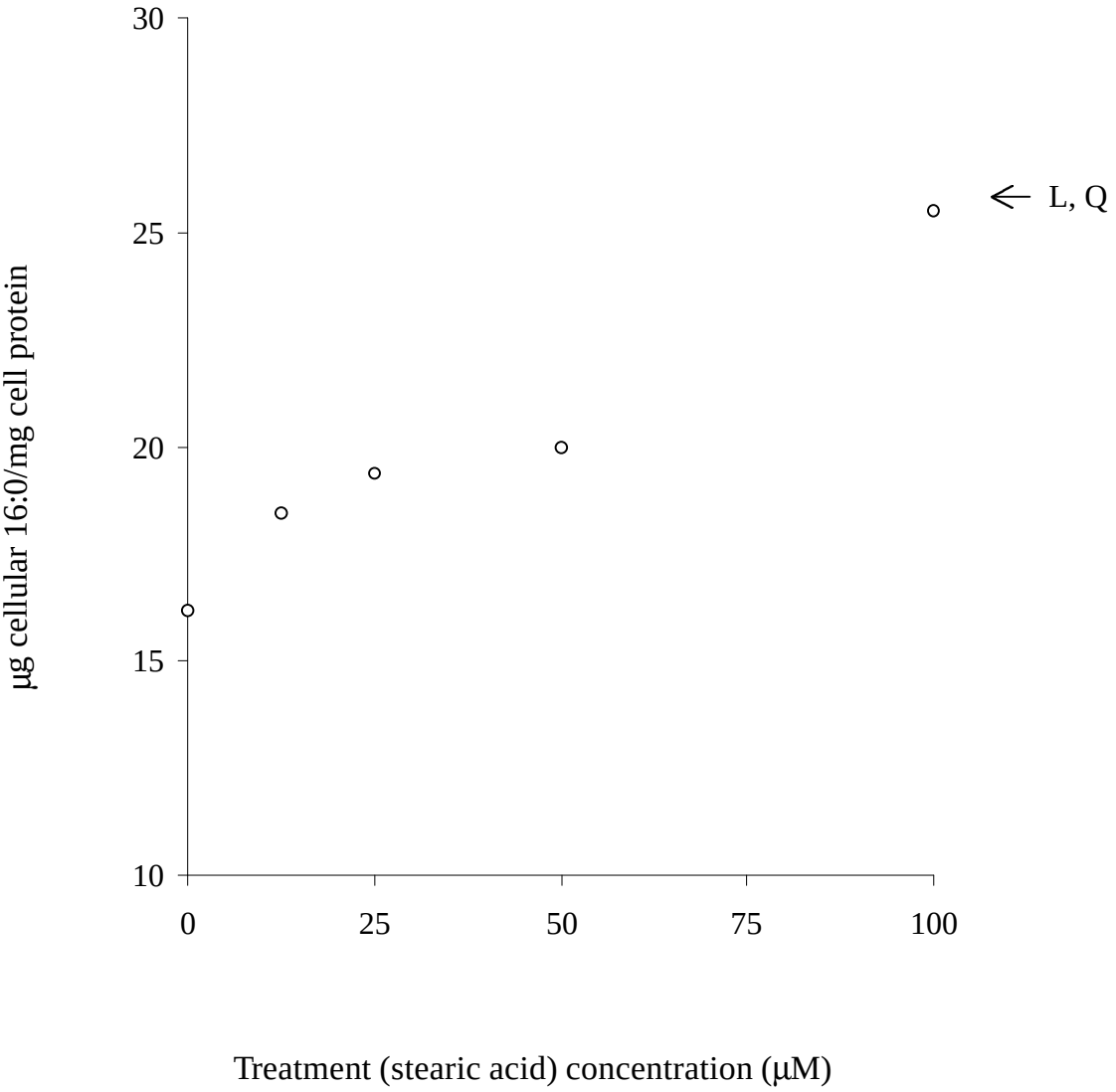

Figure 3.2
Cellular 16:0 content (µg/mg protein) in COMMA-D/MME cells in response to stearic acid treatment. L indicates significant linear response and Q indicates significant quadratic response.

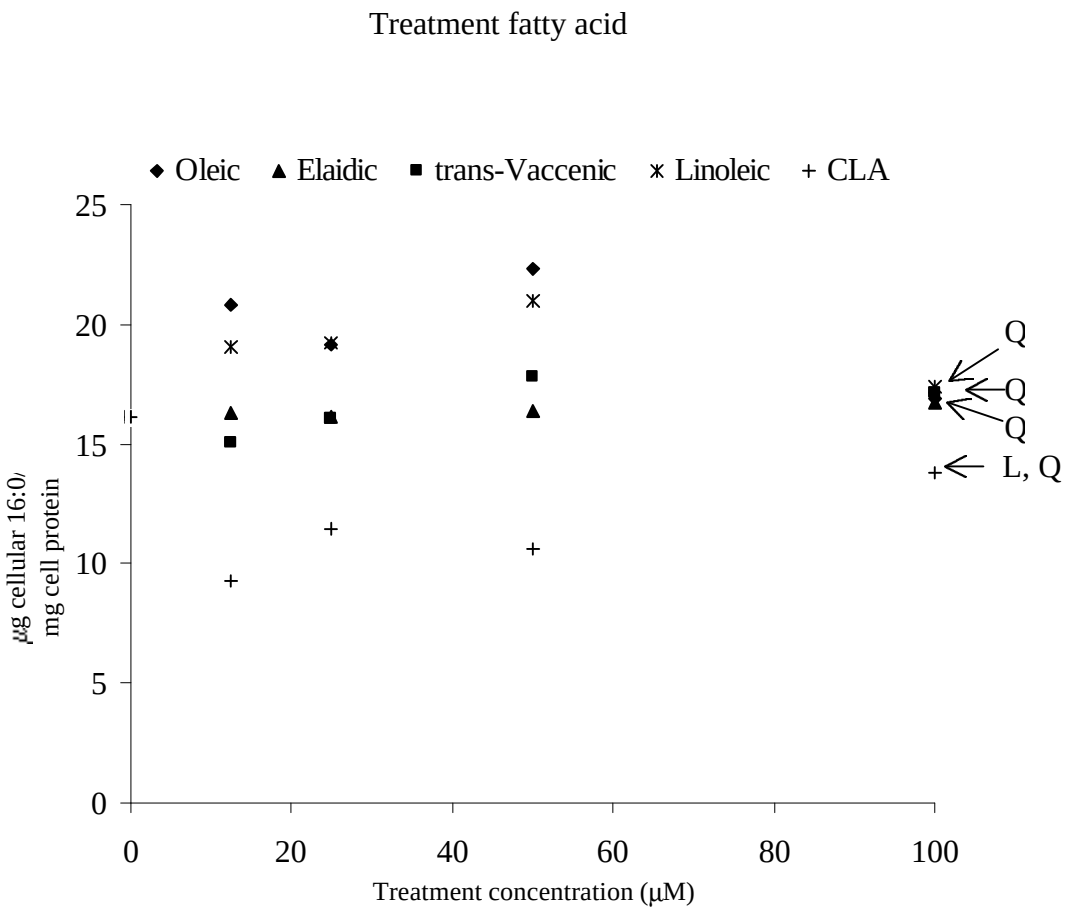

Figure 3.3
Cellular 16:0 content (µg/mg protein) in COMMA-D/MME cells in response to treatment with unsaturated fatty acids. L indicates significant linear response and Q indicates significant quadratic response.

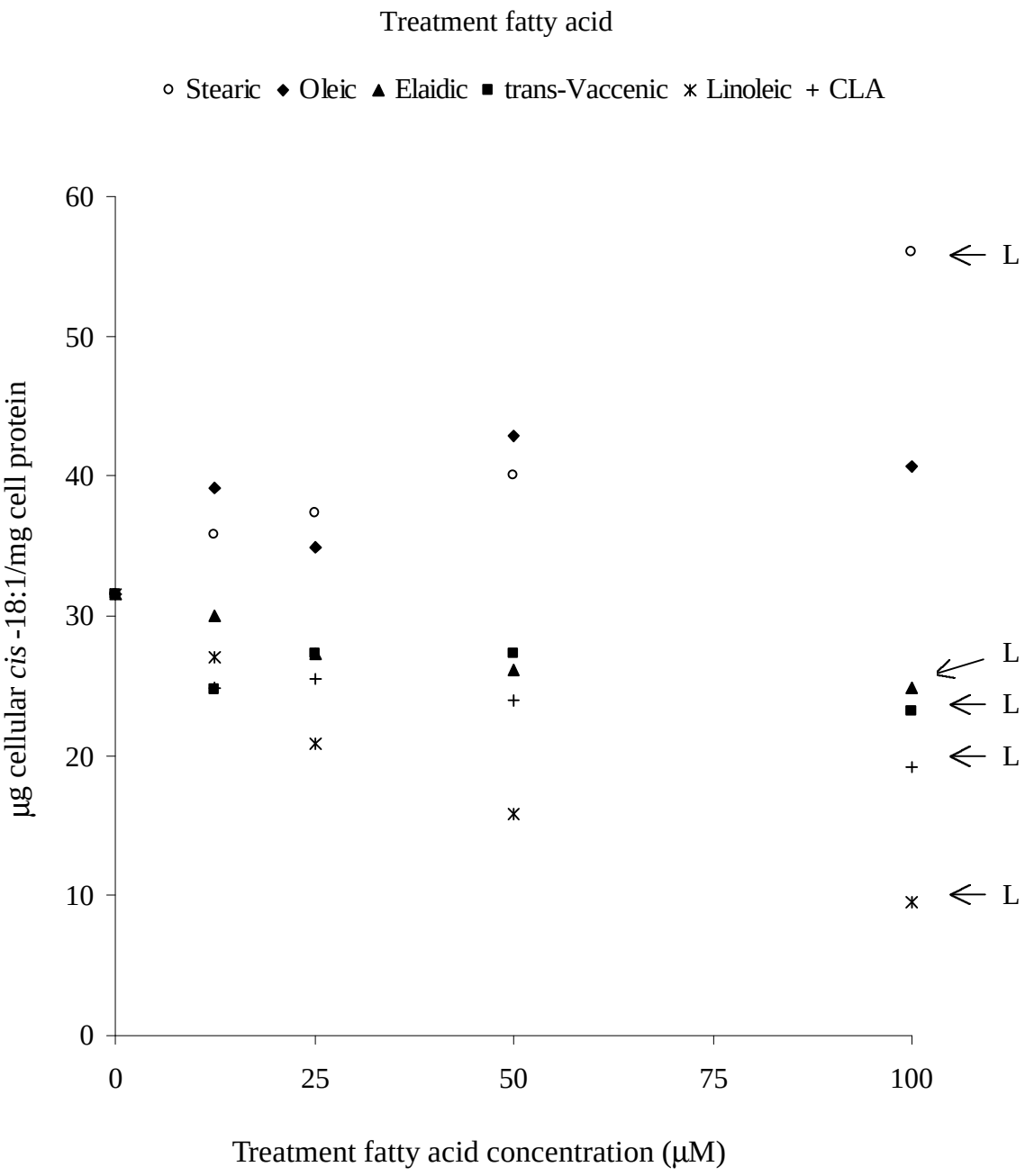

Figure 3.4
Cellular *cis*-18:1 content (μg/mg cell protein) in COMMA-D/MME cells in response to fatty acid treatment. L indicates significant linear response

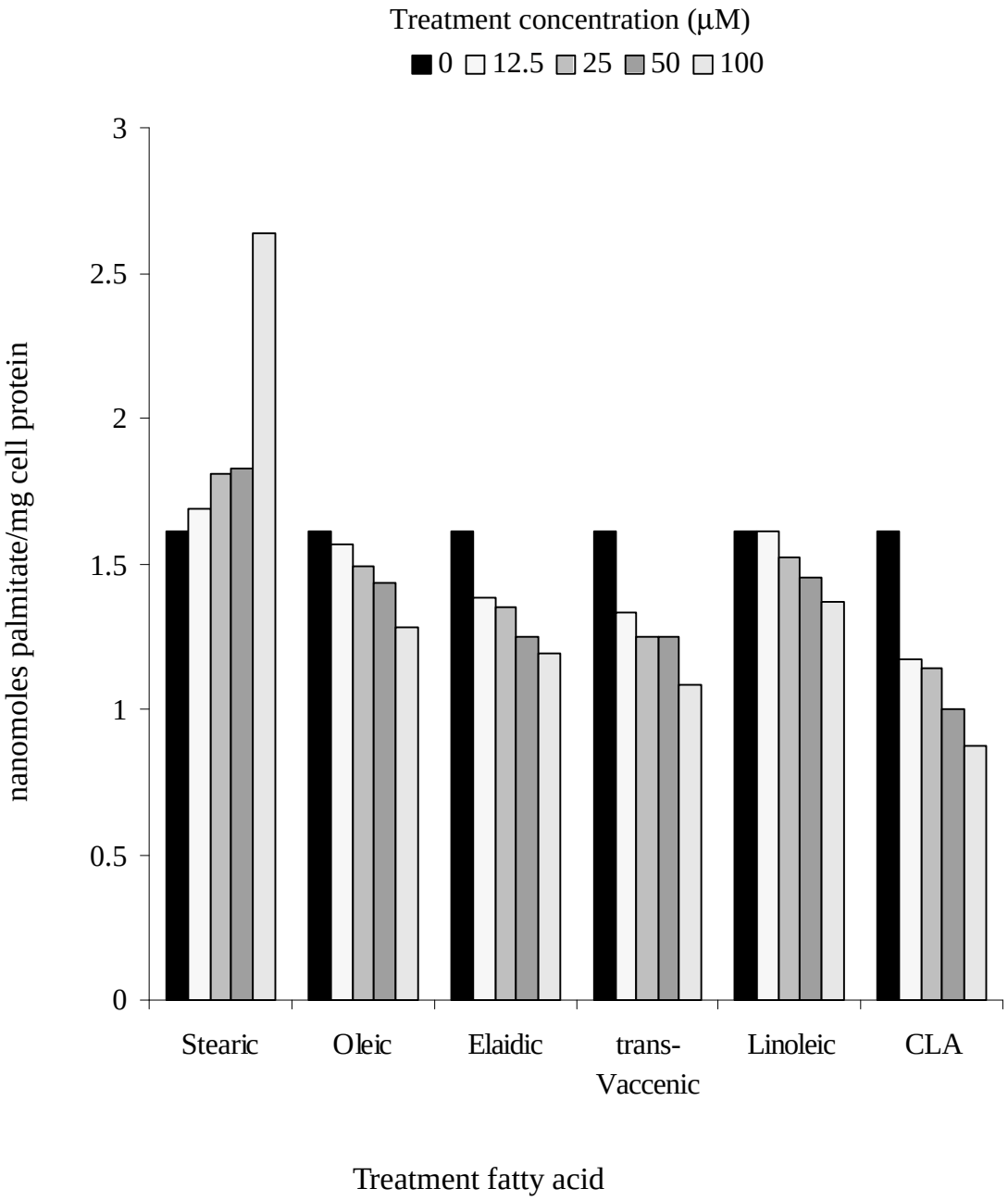

Figure 3.5
Cellular fatty acid synthetase activity (expressed as nanomoles palmitate formed from 2-^{14}C-malonyl-CoA per mg protein) in COMMA-D/MME cells in response to fatty acid treatment.

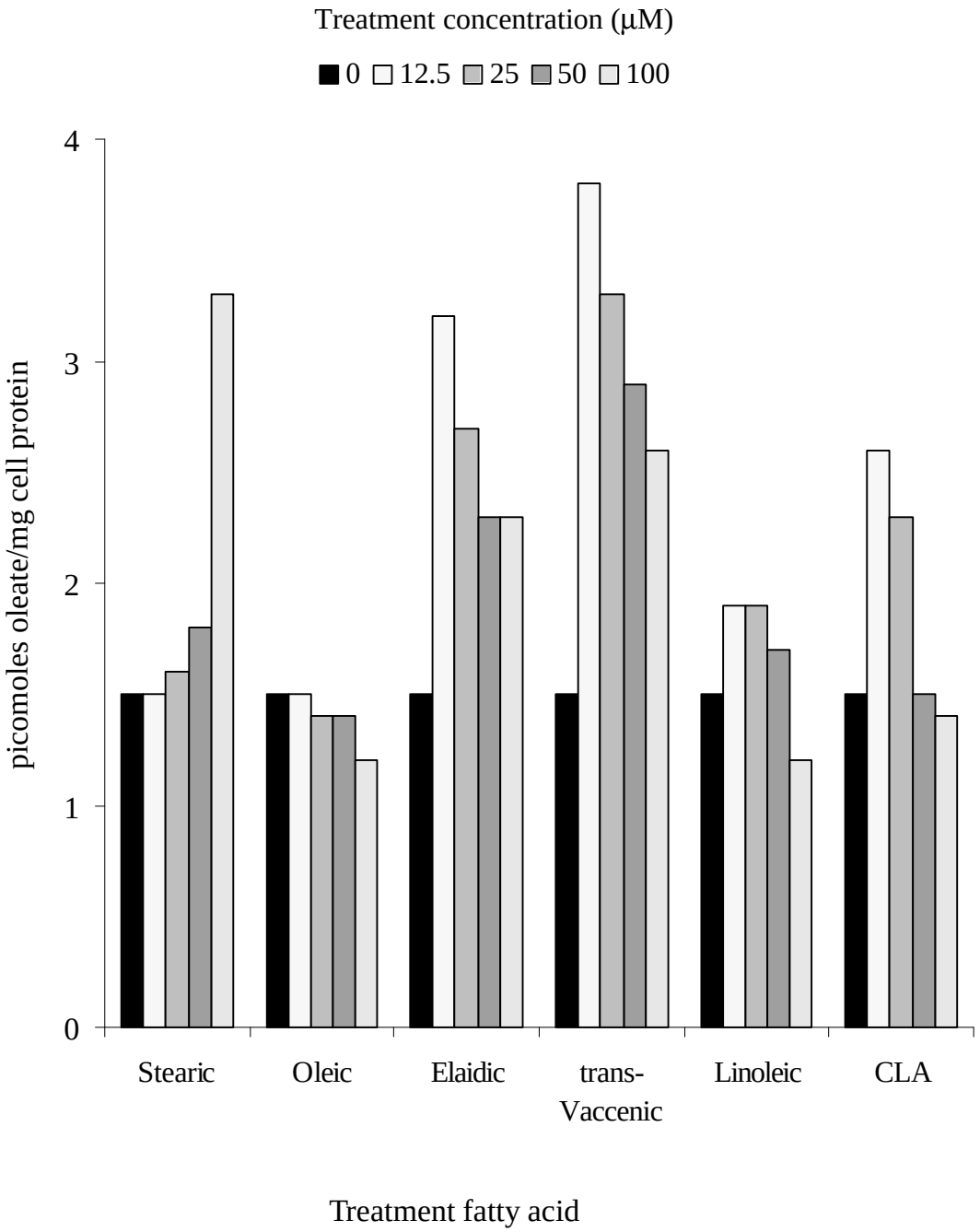

Figure 3.6
Cellular stearoyl-CoA desaturase activity (expressed as picomoles oleate formed form ^{14}C-stearoyl-CoA per mg protein) in COMMA-D/MME cells in response to fatty acid treatment.

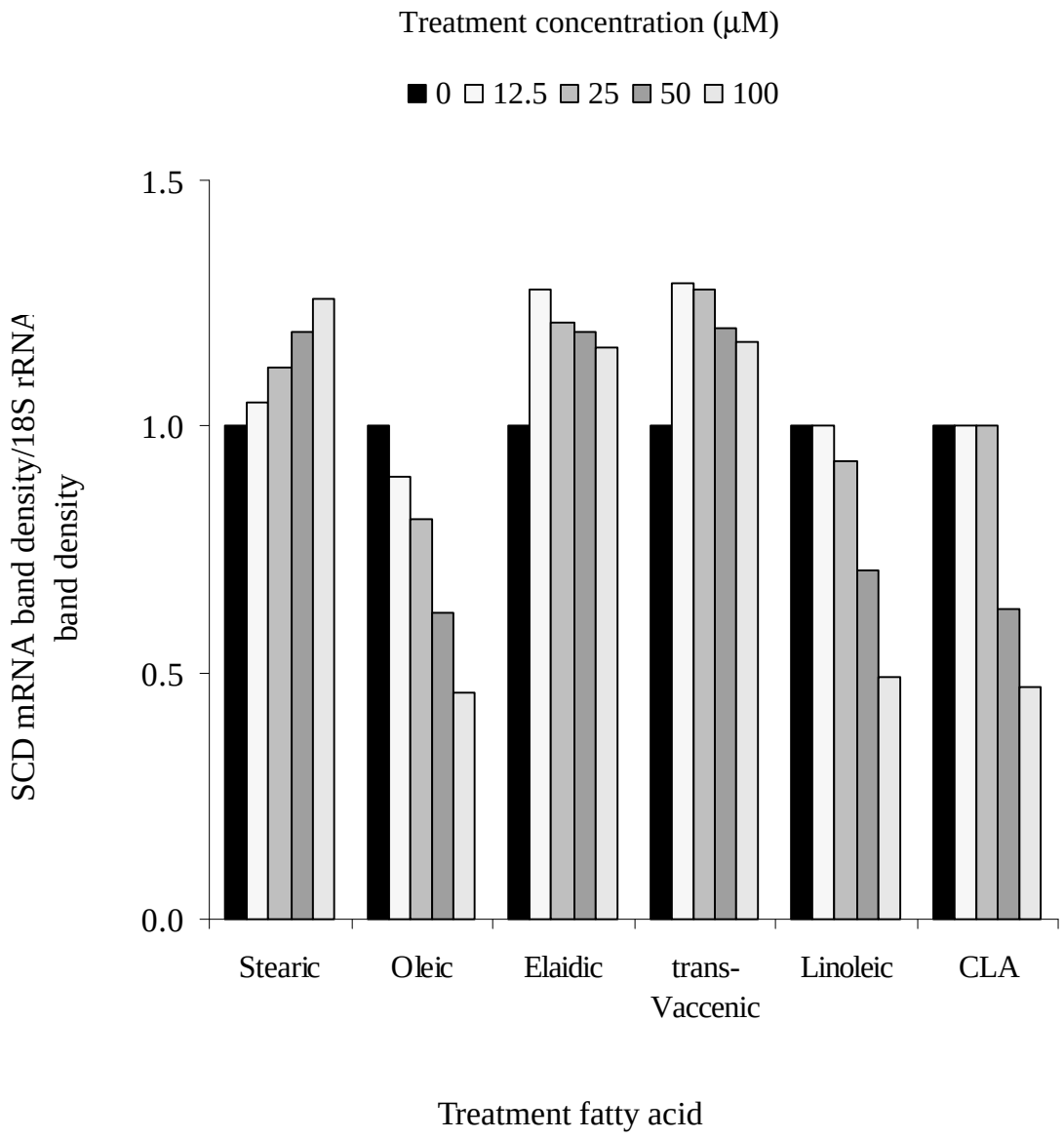

Figure 3.7
Stearoyl-CoA desaturase (SCD) mRNA abundance (expressed as ratio of density of SCD mRNA band to density of 18S rRNA band) in COMMA-D/MME cells in response to fatty acid treatment.