ELSEVIER

Effect of sterols and fatty acids on growth and triglyceride accumulation in 3T3-L1 cells

Atif B. Awad, Lina A. Begdache, and Carol S. Fink

Department of Physical Therapy, Exercise and Nutrition Sciences, State University of New York at Buffalo, Buffalo, NY, USA

Epidemiologic studies suggest a role of dietary fat in the development of obesity. Populations that consume Western diets have a higher incidence of obesity than do those that consume a vegetarian type diet such as Asians. Because dietary fats are made up mostly of triglyceride with minor lipids such as sterols, the objective of this study was to examine the effect of different fatty acids, the main component of triglycerides, and sterols on cell growth and triglyceride accumulation in 3T3-L1 cells. These cells are being used as an in vitro model for studying obesity because upon differentiation in culture they accumulate triglycerides. Cells were seeded at 5,000 cells/cm^2 and supplemented with 0, 3, 10, or 30 μM of oleic acid, elaidic acid, or docosahexaenoic acid (DHA). Similarly, cells were supplemented with 0, 2, 8, or 16 μM of cholesterol, β-sitosterol (SIT), or campesterol. Cell growth was measured by cell counting. Cellular triglycerides were measured by the Oil Red O method. In some experiments, fatty acids were combined with sterols and growth and triglyceride content were assessed as described. Both DHA and SIT had inhibitory effects on 3T3-L1 cell growth. However, SIT was more potent than DHA in this regard. The combination of SIT and oleic acid was the most potent in inhibiting cell growth and increasing cellular triglyceride content. It is concluded that cell growth and triglyceride accumulation in 3T3-L1 cells is influenced by fatty acid and sterols. When used alone, DHA and SIT inhibit cell growth. SIT was more effective in this process than was DHA. There was an interaction between fatty acids and sterols. The most effective combination inhibiting cell growth and triglyceride concentration was the combination of SIT and oleic acid. This combination reduced cell growth and increased triglyceride accumulation. These data suggest that diets rich in both monounsaturated fatty acids and phytosterols may play a role in controlling obesity. (J. Nutr. Biochem. 11:153–158, 2000)

Keywords: obesity; phytosterols; β-sitosterol; fatty acids; differentiation; triglyceride

Introduction

The prevalence of overweight and obesity cases in Western societies compared with Asians and vegetarians is strikingly high.[1] It has been estimated that obesity is responsible for approximately 5% of the total health cost in the United States.[2,3] Epidemiologic studies suggest a role of diet in the etiology of disease.[4] One of the differences in the diets consumed by societies with different incidences of obesity is the composition of dietary lipid, including fatty acids and sterols. Although some researchers have examined the role of fatty acid composition in obesity with conflicting results,[5,6] the effect of sterols has never been investigated. Compared with vegetarians and Asians, Western societies consume diets rich in saturated fatty acids but low in n-3 fatty acids. On the other hand, vegetarian and Asian diets are richer in phytosterols (PS).[7] As plant components, PS are the counterparts of cholesterol in animal tissues and are composed mainly of β-sitosterol (SIT), campesterol, and stigmasterol. Previous work from our laboratory indicated that SIT inhibits the growth of colon, prostate, and breast cancer cell lines in culture.[8–10] We[11] and others[12] have demonstrated that in vivo PS feeding normalized the hyperproliferative state of rat and mouse colonocytes, respectively. It was the purpose of the present study to investigate the effect of sterol and fatty acid composition on the growth and triglyceride accumulation in 3T3-L1 cells. As these

Address correspondence to Dr. Atif B. Awad, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214.
Received September 8, 1999; accepted December 10, 1999.

655 Avenue of the Americas, New York, NY 10010
0955-2863/00/$–see front matter
PII S0955-2863(99)00087-X

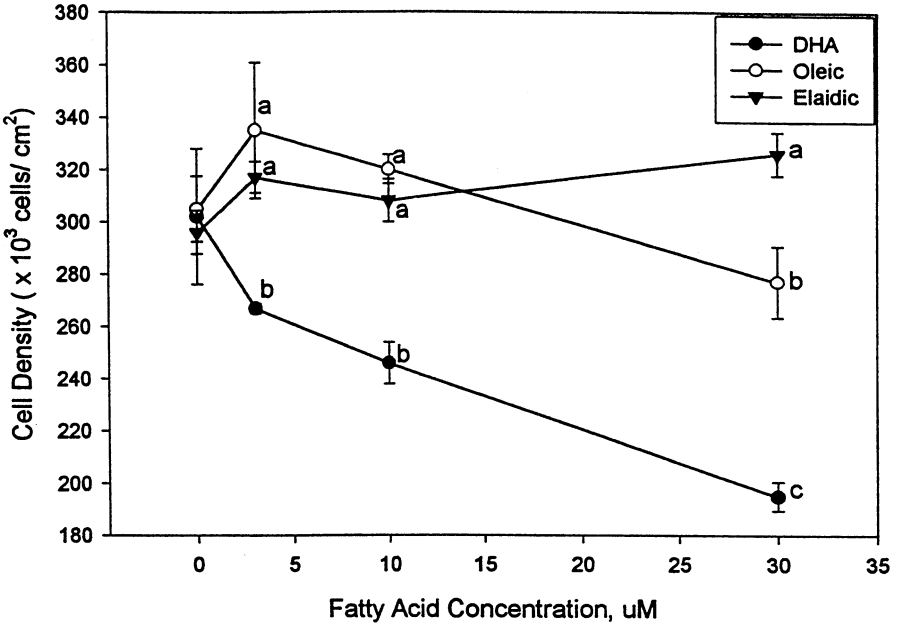

Figure 1 Effect of fatty acid supplementation on 3T3-L1 preadipocyte growth. Cells were seeded at 5,000 cells/cm^2 and supplemented with 0 (control), 3, 10, and 30 μM of oleic acid, elaidic acid, or docosahexaenoic acid (DHA). Fatty acids were supplied loaded on albumin. The controls received the vehicle (albumin). Each point represents the average of four wells ± SEM. Values with different letters in one concentration are significantly different ($P < 0.05$).

cells differentiate, triglyceride accumulates. Three sterols (cholesterol, SIT, and campesterol) and three fatty acids [oleic acid, elaidic acid, and docosahexaenoic acid (DHA)], were used in this study. Cholesterol was used to represent the main sterol in the Western diet, whereas the other sterols represent PS in Asian and vegetarian diets. Oleic acid and its trans-isomer elaidic acid are common fatty acids in Western diets whereas DHA represents an example of an omega-3 fatty acid that is usually higher in Asian and vegetarian diets, specifically in those who consume fish. The use of 3T3-L1 cells in this study offers a unique model to examine these effects in a well-controlled conditions.[13]

Methods and materials

Materials

3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD USA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin, bovine serum albumin (BSA), Oil Red O, oleic acid, elaidic acid, DHA, SIT, cholesterol, and campesterol were obtained from Sigma Chemical Co. (St. Louis, MO USA). Fetal bovine serum (FBS) and antibiotics were obtained from GibcoBRL (Grand Island, NY USA). The 24-well tissue culture plates, as well as the 25 cm^2 vented tissue Falcon culture flasks, were purchased from Becton Dickson Laboratories (Lincoln Park, NJ USA).

Methods

Cell culture. 3T3-L1 cells were subcultured and grown in DMEM containing 10% FBS, 1% antibiotic/antimycotic (10,000 U penicillin, 10 mg streptomycin, and 25 mg amphotericin B/mL), 2.2 g/L sodium bicarbonate, 4.5 g/L glucose, pH 7.3, in 25 cm^2 vented tissue culture flasks in a 37°C, 5% carbon dioxide/95% air humidified atmosphere. After seeding, cultures were left undisturbed for 24 hours to allow for attachment to the flask prior to the first feeding. Cells were fed every other day thereafter and were passed weekly. In all experiments, 3T3-L1 cells were seeded at a density of 5,000 cells/cm^2. Experiments were carried out using cells from pass 8 to 22.

Stock solutions of tested lipids (fatty acids and sterols) were prepared in ethanol. Fatty acids were delivered to the cells as fatty acid/BSA complexes as described by Murphy et al.[14] The molar ratio of fatty acid to BSA was 3:1. Sterols were delivered as sterol/cyclodextrin complexes.[8,15] The cyclodextrin used was 2-hydroxypropyl-β-cyclodextrin (CD; Sigma Chemical Co.). The final concentration of CD in the media was 5 mM. The concentration of fatty acids used ranged from 3 to 30 μM and that of sterols ranged from 2 to 16 μM. Cell growth was monitored by cell counting using a Coulter counter (Hialeah, FL). The coefficient of variability of cell counting using this instrument is below 1%. For examining the interaction between the two lipids, the controls received both BSA and CD. Each experiment was repeated twice to assure reproducibility.

Cell triglyceride measurement. The Oil Red O method of Ramirez-Zacarias et al.[16] was used to measure triglyceride content. A triolein standard was used to establish the standard curve.

Statistical analysis

Data were analyzed by analysis of variance and the differences between the groups were tested for significance by the post-hoc Newman-Keuls test. The statistical software used was SigmaStat (Jandel Scientific, San Raphael, CA USA). The differences between the means were considered statistically significant if the *P*-value was less than 0.05.

Results

Effect of fatty acids on cell growth

Figure 1 depicts the effect of different fatty acids at three concentrations on cell growth for 5 days. There was no significant difference between the growth of cells fed either oleic or elaidic acids at 3 or 10 μM concentrations. However, elaidic acid at 30 μM supported cell growth much better than did oleic acid. At all concentrations, DHA inhibited the growth compared with the monounsaturated

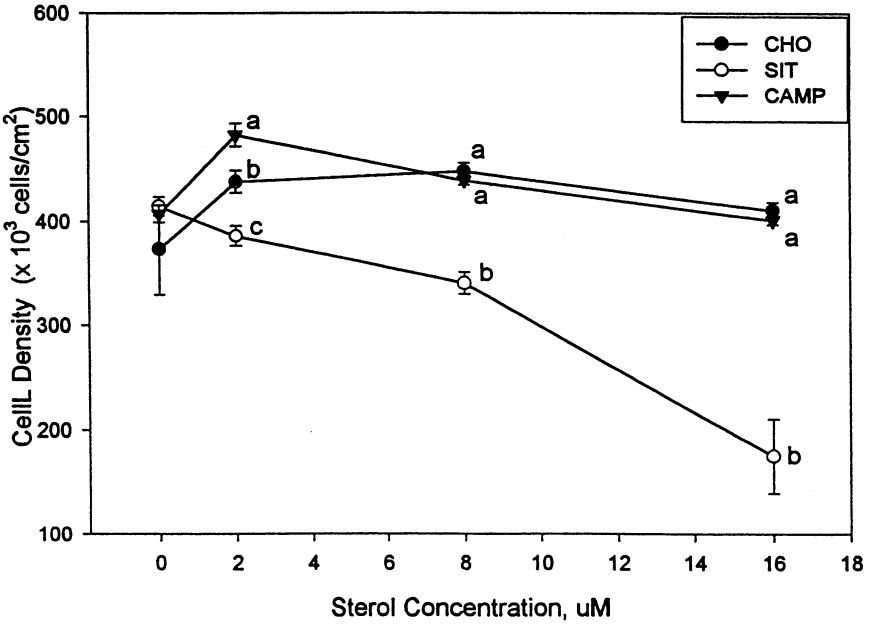

Figure 2 Effect of sterol supplementation on 3T3-L1 preadipocyte growth. Cells were seeded at 5,000 cells/cm^2 and supplemented with 0 (control), 2, 10, and 16 μM of cholesterol (CHO), sitosterol (SIT), or campesterol (CAMP). Sterols were supplied loaded on cyclodextrin (5 mM). The controls received the vehicle (cyclodextrin). Each point represents the average of four wells ± SEM. Values with different letters in one concentration are significantly different ($P < 0.05$).

fatty acids tested. This inhibition was approximately 24% at 10 μM but 31 to 40% at 30 μM.

Effect of sterols on cell growth

Figure 2 demonstrates the effect of the sterols supplemented for 5 days on cell growth. Campesterol at 2 μM supported the growth much better than did cholesterol. However, at higher concentrations no differences between campesterol and cholesterol were observed. At all concentrations, SIT supplementation resulted in a significant reduction in growth of 3T3-L1 cells. The reduction was 12 to 19% at 2 μM, 24% at 8 μM, and 65% at 16 μM.

Effect of combining sterols and fatty acids on the growth of 3T3-L1

Studies on the effect of fatty acids and sterols on cell growth indicated that elaidic acid and campesterol had no effect on growth compared with oleic acid and cholesterol, respectively. The combined experiment concentrated on only two fatty acids (oleic acid and DHA) and two sterols (cholesterol and SIT) in studying the interaction between fatty acids and sterols on cell growth. The concentrations of lipids used in these experiments were either one half the maximum effective doses or the maximum effective doses so that we could detect any synergistic or additive effect, if any, on cell growth. *Figure 3* depicts this effect when 8 μM sterol was used in combination with 16 μM fatty acid. The control group received the two vehicles. Cholesterol/

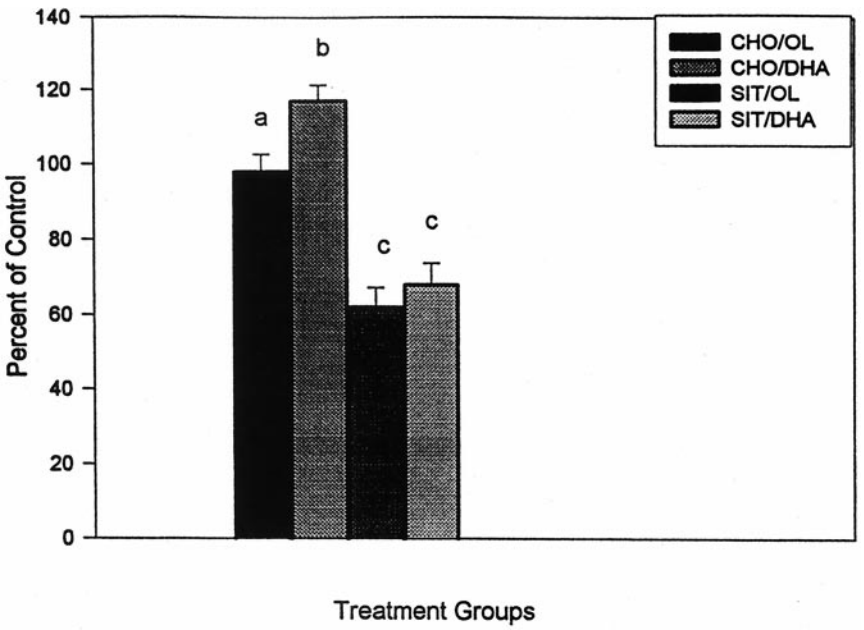

Figure 3 Effect of 15 μM fatty acids/8 μM sterol on 3T3-L1 growth. Cells were seeded at 5,000 cells/cm^2 and supplemented with cholesterol (CHO)/oleic acid (OL), CHO/docosahexaenoic acid (DHA), sitosterol (SIT)/OL or SIT/DHA. The controls received both vehicles, 5 mM cyclodextrin, and 10 μM bovine serum albumin (BSA). Fatty acid concentration was kept at 15 μM, sterols at 8 μM, cyclodextrin at 5 mM, and BSA at 10 μM. Each bar represents the average of six observations ± SEM. Values with different letters in one concentration are significantly different ($P < 0.05$).

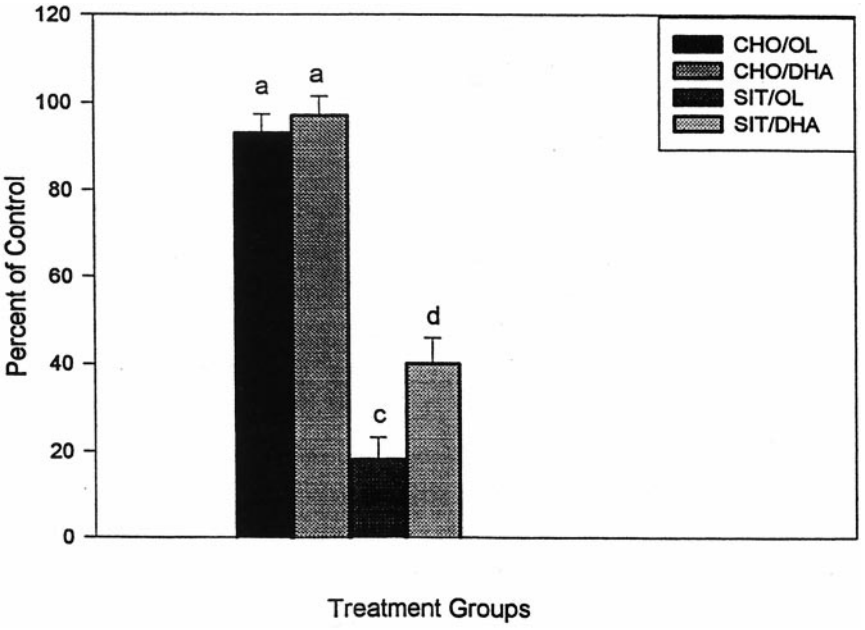

Figure 4 Effect of 30 μM fatty acids/16 μM sterol on 3T3-L1 growth. Cells were seeded at 5,000 cells/cm^2 and supplemented with cholesterol (CHO)/oleic acid (OL), CHO/docosahexaenoic acid (DHA), sitosterol (SIT)/OL or SIT/DHA. The control received vehicles, 5 mM cyclodextrin (CD), and 10 μM BSA. Fatty acid concentration was kept at 30 μM, sterols at 16 μM, CD at 5 mM, and BSA at 10 μM. Each bar represents the average of six observations ± SEM. Values with different letters are significantly different ($P < 0.05$).

oleic acid fed cells had growth similar to that of the control. Cholesterol/DHA had 20% greater growth than the control. Both SIT/oleic acid and SIT/DHA supplemented cells grew similarly at 62 to 69% that of cholesterol/oleic acid supplemented cells.

When the previous experiment was repeated using double the concentration of lipids (*Figure 4*), we noticed that cholesterol fed cells, regardless of the type of fatty acids, had growth similar to that of the control. On the other hand, cells that received SIT had lower growth; the lowest was in cells supplemented with oleic acid compared with DHA.

Effect of sterol and fatty acid supplementation on triglyceride accumulation by 3T3-L1 cells

Because one of the main objectives of this study was to examine the combined effect of fatty acids and sterols on triglyceride accumulation in 3T3-L1 cells, we used the maximum lipid doses identified in these studies, 30 μM for fatty acids and 16 μM for sterols.

Figure 5 demonstrates that the greatest accumulation of triglycerides occurred when cells were supplemented with SIT in the presence of oleic acid (82% more) and the least was when cells were supplemented with cholesterol and DHA.

Discussion

The present studies generated three interesting findings. First, we have shown that SIT, which is the most abundant dietary PS, inhibits cell growth and stimulates triglyceride accumulation in 3T3-L1 cells. This mouse cell line is widely accepted as a model for the study of obesity,[17] because it exhibits many mammalian adipocyte characteristics such as

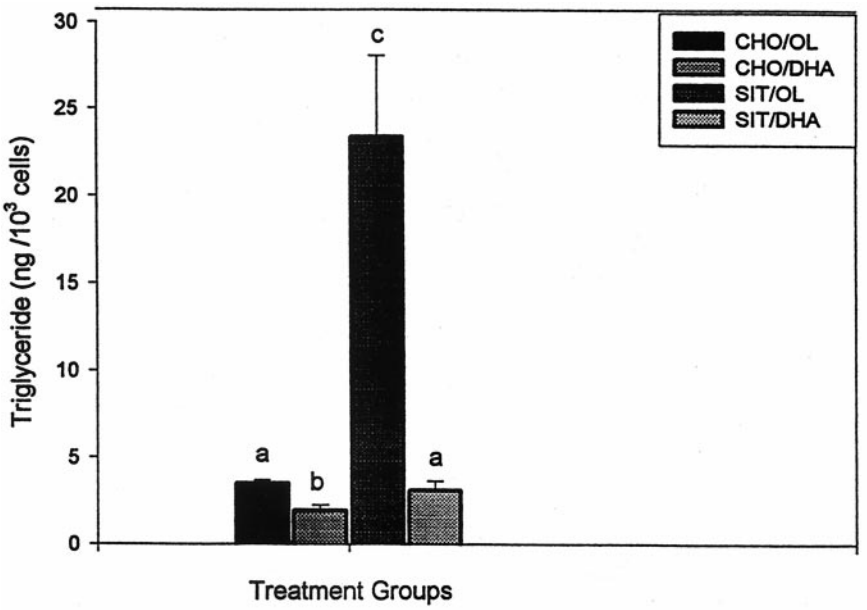

Figure 5 Effect of 30 μM fatty acids/16 μM sterol on 3T3-L1 triglyceride concentration. Cells were fixed with 1 mL of formalin for 1 hour and stained for 2 hours with Oil Red O. Each bar represents the average of 6 wells ± SEM. Values with different letters are significantly different ($P < 0.05$). CHO, cholesterol; SIT, sitosterol; OL, oleic acid; DHA, docosahexaenoic acid.

cellular differentiation and lipid accumulation. Second, the present study identified DHA as a fatty acid that reduces cell growth and accumulates triglycerides in 3T3-L1 cells. Third, the combination of the most effective doses of fatty acids and sterols on cell growth and triglyceride accumulation revealed interactions of these lipids that may have implications on the development of obesity. Epidemiologic studies suggest that obesity is more prevalent in Western societies than in vegetarians and Asian populations.[2,18]

Using fatty acid concentrations within physiologic levels,[19] the present study showed that oleic acid and its trans-isomer elaidic acid have no significant effect on cell growth. On the other hand, omega-3 fatty acids such as DHA inhibited the growth of 3T2-L1 cells compared with monounsaturated (omega-9) fatty acids. This was consistent with the finding of many studies that showed that polyunsaturated fatty acids have antiproliferative properties on different types of cells.[20,21] Some short-term studies revealed that there is no effect of fatty acid composition on body weight,[5] whereas other feeding studies showed that there was an effect on the development of obesity.[22]

The second important finding is the inhibitory effect of SIT on the growth of 3T3-L1 cells. To the best of our knowledge, this is the first report on the effect of sterols on cell growth and triglyceride accumulation in 3T3-L1 cells. Our data shows that SIT, within the physiologic levels seen in the blood of vegetarians,[23] inhibits cell growth compared with campesterol, which is the less common dietary PS, or cholesterol, which is the predominant sterol in the Western diet. A similar effect of SIT was detected on the growth of tumor cells such as HT-29,[8] LNCaP,[9] and MDA-MB-231.[10]

When the most effective doses of SIT and DHA were used in combination, no synergistic or additive effects on cell growth and triglyceride accumulation was noticed. This suggests that DHA and SIT might influence these parameters by different mechanisms. Several theories concerning the effect of DHA on cell membrane functions have been offered. Polyunsaturated fatty acids are know to be susceptible to peroxidation, particularly DHA because it contains six double bonds.[24] It is speculated that fatty acid peroxidation may cause the release of free radicals, which might be toxic to the cell. In addition, because membrane functions are influenced by membrane fluidity, dietary polyunsaturated fatty acids may also influence insulin binding to its receptors, which in turn may prevent the synthesis of lipogenic enzymes.[25]

The ring structure of sterols is believed to play a role in the incorporation of different sterols into cell membranes.[26] Cholesterol and campesterol did not show any significant effect on cell growth of 3T3-L1 cells, probably due to the close similarity in their structures. Campesterol has a methyl group on carbon 24 that allows it to be fully incorporated into cell membranes. On the other hand, SIT has an ethyl group on carbon 24, which might hinder its incorporation into the membranes, thus altering the functional properties of the cell. Some studies have shown that SIT incorporation into cell membranes results in increased membrane rigidity and an increase in the activity of delta-6-desaturase to adjust the altered fluidity.[27] Consequently, in combination with sterols, the polyunsaturated fatty acids might correct the alteration in cell membranes induced by sterol incorporation. The increase in rigidity imparted by SIT may be counteracted by the increased membrane fluidity induced by DHA supplementation. This could explain the observed increase in cell growth when DHA was used with sterols. Thus, the net effect on cell growth (cell number) would not necessarily be the sum of the individual effects of DHA and SIT. However, other mechanisms may be responsible for the decreased cell growth mediated by the combination of fatty acids and SIT. Such mechanisms could include the effect of SIT on apoptosis and sphingomyelin cycle.[9,28]

Regardless of the mechanism(s) by which these lipids affect cell growth, the present data indicate that sterols had a more pronounced effect on the growth of 3T3-L1 cells than did fatty acids. In addition, these studies demonstrated that supplementation with SIT is associated with triglyceride accumulation. However, this effect was more pronounced in cells supplemented with SIT/oleic acid than with SIT/DHA. The fact that the combination of fatty acid and SIT inhibits cell growth and increases triglycerides suggests that these lipids induce differentiation in 3T3-L1 cells. However, additional biological markers for cell differentiation must be used to confirm this point. Because leptin production is associated with triglyceride accumulation in fat cells, this suggests that SIT and DHA in combination in the diet may play a role in the development of obesity. We were not able to measure leptin in cells in these experiments regardless of the triglyceride accumulation (unpublished work). Studies using 3T3-L1 preadipocytes detected leptin mRNA using specific differentiating agents in culture only after a prolonged period.[29] Studies are underway in our laboratory to examine the effect of SIT and DHA, alone or in combination, on the expression of leptin by these cells after full differentiation.

In conclusion, our data show that sterols and fatty acids have significant effects on cell growth and triglyceride accumulation in 3T3-L1 preadipocytes. SIT was found to be the most effective growth inhibitor among the sterols and DHA among the fatty acids studied. Furthermore, the results revealed that there was an interaction between the two lipids tested (sterols and fatty acids), which was demonstrated by different effects on growth and triglyceride accumulation when supplemented in combination to the 3T3-L1 cells. However, the present study did not reveal the details of this interaction and further studies are needed to examine the mechanisms by which sterols and fatty acids affect 3T3-L1 cell growth and triglyceride accumulation.

Acknowledgment

The authors would like to thank Kathleen Galas for typing the manuscript. The research was supported by a grant from the Allen Foundation.

References

1 Colditz, G.A. (1992). Economic costs of obesity. *Am. J. Clin. Nutr.* **55,** 503S–507S

2 Barnard, N.D., Nicholson, A., and Howard, J.L. (1995). The medical costs attributable to meat consumption. *Prev. Med.* **24,** 646–655

3 Curhan, G.C., Willett, W.C., Rimm, E.B., Spiegelman, D., Ascherio, A.L., and Stampfer, M.J. (1996). Birth weight and adult hyperten-

sion, diabetes mellitus, and obesity in U.S. men. *Circulation* **94,** 3246–3250
4 Lee, R.D. and Nieman, D.C. (1996). *Nutritional Assessment*, 2nd ed. Mosby-Year Book, Inc., St. Louis, MO USA
5 Awad, A.B., Bernardis, L.L., and Fink, C.S. (1990). Failure to demonstrate an effect of dietary fatty acid composition on body weight, body composition and parameters of lipid metabolism in mature rats. *J. Nutr.* **120,** 1277–1282
6 Shimomura, Y., Tamura, T., and Suzuki, M. (1990). Less body fat accumulation in rats fed a safflower oil diet than in rats fed a beef tallow diet. *J. Nutr.* **120,** 1291–1296
7 Messina, M. and Barnes, S. (1991). The role of soy products in reducing the risk of cancer. *J. Natl. Cancer Inst.* **83,** 541–546
8 Awad, A.B., Chen, Y.C., Fink, C.S., and Hennessey, T. (1996). β-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. *Anticancer Res.* **16,** 2797–2804
9 von Holtz, R.L., Fink, C.S., and Awad, A.B. (1998). β-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. *Nutr. Cancer* **32,** 8–12
10 Downie, A., Fink, C.S., and Awad, A.B. (1999). Effect of phytosterols on MDA-MB-231 breast cancer cell growth. *FASEB J.* **13,** A433.3
11 Awad, A.B., Tagle-Hernandez, A.T., Fink, C.S., and Mendel, S.L. (1997). Effect of dietary phytosterols on cell proliferation and protein kinase C activity in rat colonic mucosa. *Nutr. Cancer* **27,** 210–215
12 Janezic, S.A. and Rao, A.V. (1992). Dose-dependent effect of dietary phytosterols on epithelial cell proliferation of the murine colon. *Food Chem. Toxicol.* **30,** 611–616
13 Green, H. and Kehinde, O. (1976). Spontaneous heritable changes leading to adipose conversion in 3T3 cells. *Cells* **7,** 105–113
14 Murphy, S., Albright, E., Marthur, S.N., and Field, F.J. (1988). Modifications of Caco-2 cell membrane fatty acid composition by eicosapentaenoic acid and palmitic acid: Effect of cholesterol metabolism. *J. Lipid Res.* **29,** 773–780
15 Greenburg-Ofrath, N., Terespolosky, Y., Kahane, I., and Bar, R. (1993). Cyclodextrins as carriers of cholesterol and fatty acids in cultivation of mycoplasms. *Appl. Envir. Micro.* **59,** 547–551
16 Ramirez-Zacarias, J.L., Castro-Munozledo, F., and Kuri-Harcuch, W. (1992). Quantification of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil Red O. *Histochemistry* **97,** 493–497
17 Green, H. and Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipids. *Cell* **1,** 113–116
18 Key, T. and Davey, G. (1996). Prevalence of obesity is low in people who do not eat meat. *Br. Med. J.* **313,** 816–817
19 Montgomery, R., Conway, T.W., and Spector, A.A. (1990). *Biochemistry, A Case Oriented Approach*. *Mosby Corp*., St. Louis, MO USA
20 Coleman, P.S., Chen, L.C., and Sepp-Lorenzino, L. (1997). Cholesterol metabolism and tumor cell proliferation. *Sub-Cellular Biochem.* **28,** 363–435
21 Grammatikos, S.I., Subbaiah, P.V., Vistor, T.A., and Miller, W.M. (1994). n-3 and n-6 fatty acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial cell lines. *Br. J. Cancer* **70,** 219–227
22 Carlotti, M., Hainault, I., Guichard, C., Hadjuch, E., and Lavau, M. (1993). Fish oil in a high lard diet prevents obesity, hyperlipemia and adipocyte insulin resistance in rats. *Ann. NY Acad. Sci.* **683,** 98–101
23 Salen, G., Ahrens Jr., E.N., and Grundy, S.M. (1970). Metabolism of β-sitosterol in man. *J. Clin. Invest.* **49,** 952–967
24 Begin, M.E., Ells, G., and Horrobin, D.F. (1988). Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. *J. Natl. Cancer Inst.* **80,** 188–194
25 Thompson, M.P. (1996). Meal-feeding specifically induces obese mRNA expression. *Biochem. Biophys. Res. Commun.* **224,** 332–337
26 Child, P. and Kuksis, A. (1983). Critical role of ring structure in the differential uptake of cholesterol and plant sterols by membrane preparations in vitro. *J. Lipid Res.* **24,** 1196–1209
27 Hennessey, T.M. (1992). Effects of membrane plant sterols on excitable cell functions. *Comp. Biochem. Physiol.* **101C,** 1–8
28 Awad, A.B., von Holtz, R.L., Cone, J.P., Fink, C.S., and Chen, Y.-C. (1998). β-Sitosterol inhibits the growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle. *Anticancer Res.* **18,** 487–479
29 McDougald, O.A., Hwang, C.S., Fan, H., and Lane, M.D. (1995). Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA* **92,** 9034–9037