340

Down-regulation of Fatty Acid Synthase Is Associated with Decreased Akt Activation in Lovastatin Induced Apoptosis Cells

YUAN-CHING CHANG[1,2], YEN-HUA HUANG[3], CHWEN-MING SHIH[3], JUI-YU WU[3], CHIEN-LIANG LIU[1,2], SHWU-HUEY WANG[4] AND CHUN-MAO LIN[3,4]*

[1.] *Department of General Surgery, Mackay Memorial Hospital, Taipei City, Taiwan*

[2.] *Mackay Medicine, Nursing and Management College, Peito, Taipei City, Taiwan*

[3.] *College of Medicine, Taipei Medical University, Taipei City, Taiwan*

[4.] *Instrumentation Center, Taipei Medical University, Taipei City, Taiwan*

ABSTRACT

Increased fatty acid synthase (FAS) protein expression is coordinated with cancer development. FAS inhibitors become a focus of anticancer drug development. Lovastatin, one of the active ingredients in red yeast rice, is a product of *Monascus purpureus*. Lovastatin has been shown to inhibit proliferation and to induce apoptosis in a variety of tumor cells. This report shows that chemopreventive effects of lovastatin may be through the down regulation of FAS. Lovastatin exhibited significant apoptosis-inducing activity in HL-60 cells, as observed by flow cytometry (with 49.83% in sub-G1 peak compared to the control, 10.43%), blebbing cell membrane morphology, and nuclear condensation. Cellular triglyceride, cholesterol, and free fatty acid in HepG2 cells were reduced to 79%, 81%, and 75%, respectively, upon lovastatin treatment (50 μM) for 4 h. The relative levels of FAS protein after treatment with 0, 10, 20, and 50 µM lovastatin were 1.00, 0.89, 0.72, and 0.31, respectively. Phosphorylated Akt was reduced in a dose-dependent manner. Reverse transcription PCR analysis showed that lovastatin upregulated PPAR-γ and inhibited SREBP-1 mRNA expression in HepG2 cells. Our current results implicate that lovastatin inhibiting FAS expression is associated with the decreased Akt activation.

Keywords: fatty acid synthase, lovastatin, PKB/Akt, PPAR, statin, SREBP

INTRODUCTION

Red yeast rice, an asian dietary staple made by fermenting red yeast (*Monascus purpureus*) on rice, gains rapid recognition as a cholesterol-lowering agent[1]. This is because a careful fermentation process yields statins, the compounds largely helping reduce total cholesterol levels, lower levels of low density lipoprotein (LDL) cholesterol and triglycerides, and increase levels of high density lipoprotein (HDL) cholesterol. Statins also exert a number of pleiotropic and vasculoprotective actions. Statins have been shown to boost cerebral blood flow and provide significant protection against stroke in animal models[2]. Recent evidence has implicated that a sudden discontinuation of statin treatment could cause a rebound effect and downregulation of NO production. Withdrawal of statin treatment may hinder vascular function and result in higher morbidity and mortality in vascular disease patients[3]. Recently, evidence has emerged that statin drug use in human patients results in significant reduction in cancer incidence[4]. Lovastatin, also known as Monacolin K, is one of the active ingredients in red yeast rice. Many studies

in *in vitro* systems and experimental animals have reported that lovastatin is an effective antitumor agent[5]. Lovastatin induces apoptosis in myeloma and lymphoma cells by inhibition of geranylgeranylation and subsequent down regulation of anti-apoptotic protein in myeloma[6].

Activated lipogenesis is a common feature of tumor cells from a variety of cancers[7]. The expression of FAS reflects the regulation of lipogenesis because FAS is the major enzyme in fatty acid biosynthesis. FAS expression has been demonstrated through phosphatidyl-inositol 3-kinase (PI3K)-Akt signaling. The role of mitogen-activated protein kinase (MAPK) on mediating FAS expression in H-ras transformation of cells has been reported[7]. Up-regulation of sterol regulatory element binding protein (SREBP) results in FAS overexpression. SREBPs, a family of transcription factors, activated genes involving in the synthesis of cholesterol and fatty acid and their uptake from plasma lipoproteins[8]. PI3K-Akt signaling represents a common downstream for regulating lipogenesis. MEK-1 crosstalks with this pathway to coordinately regulate SREBP and FAS. The present study was undertaken to investigate the chemopreventive effects of lovastatin through down regulation of FAS expression.

_____
* Author for correspondence. Tel: +886-2-27361661 ext. 3165; Fax: +886-2-27361661 ext. 3164; Email: cmlin@tmu.edu.tw

MATERIALS AND METHODS

I. *Cell Culture*

The HepG2 cells were obtained from the ATCC, and were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10% lipoprotein-deficient fetal bovine serum (LDS) (Sigma, St Louis, MO). Cells were subcultured in a 35-mm-diameter dish to about 80% confluence and supplemented with 7% LDS in RPMI-1640 (LDS-RPMI) for 16 h to induce cholesterologenesis. After induction, cells were treated with lovastatin in LDS-RPMI. Untreated control cells received 0.1% (v/v) DMSO without lovastatin.

Human promyelocytic leukemic HL-60 cells were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37°C. HL-60 cells (2×10^5 /mL) were subcultured in a 35-mm-diameter dish for drug treatment.

II. *Analysis of Cellular Triglyceride (TG), Cholesterol, and Free Fatty Acid*

To measure the cellular TG and cholesterol, treated and untreated cells were labeled with 5 mCi/mL [1,3-^{14}C]glycerol (TG) or [1-^{14}C]acetate (cholesterol) for 4 h, respectively. After labeling, the cells were washed twice with cold PBS. Cellular lipids were then extracted with hexane-isopropanol (3:2 v/v) as described by Schuster *et al*.[9]. The organic solvent was evaporated, and the residual lipid was resuspended in hexane and spotted on a TLC plate. Neutral and polar lipids were resolved using a 2-solvent system. Plates were first developed in chloroform-methanol-acetic acid-formic acid-water (70:30:12:4:2) and then in ether-diethylether-glacial acetic acid (90:10:1). The TLC plates were dried, and the lipid zones corresponding to the lipid standard were cut, mixed in scintillation cocktail, and counted on a liquid scintillation counter. Free fatty acid was measured using iodomethane (CH_3I) and solid phase extraction (SPE) cartridges as described previously[10].

III. *Western Blots*

Cellular proteins were prepared using Gold lysis buffer (10%(v/v)glycerol, 1%(v/v)Trion X-100, 1 mM sodium orthovanadate, 20 mM Tris-base, pH 7.9, 1 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 10 mM NaF, 137 mM NaCl, 5 mM EGTA, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Thirty to fifty μg proteins were separated on SDS-PAGE and electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon^P, Millipore, Bedford, MA). The membrane was incubated with primary antibody at room temperature for 2 h, and then incubated with horseradish peroxidase-conjugated secondary IgG antibody for 1h. The immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL, Amersham, Buckinghamshire, UK). The visual bands were quantified using a densitometer (Alpha Innotech IS-1000 Digital Imaging System, California)[11].

IV. *Reverse Transcription-Polymerase Chain Reaction*

Total RNA of HepG2 cells was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Five μg total RNA was reversely transcribed (RT) into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT)$_{18}$ primer by incubating the reaction mixture (15 μL) at 37°C for 90 min. The polymerase chain reaction (PCR) was performed in a final volume of 25 μL containing dNTPs (each at 200 μM), 1X reaction buffer, 0.4 μM each primer (PPAR-γ1: Forward 5'-GTTCATGCTTGT-GAAGGATGC-3', Reverse 5'-ACTCTGGGTTCAGCT-GGTCG-3'; SREBP-1c: Forward 5'-ACCCTG-GTGAGTGGAGGGACCATCTTGG-3', Reverse 5'-CTTTGCTTCAGTGCCACCACCAGGTCTTT-3'; GAPDH: 5' TGAAGGTCGGTGTGAACGGATTTGGC 3' and 5' CATGTAGGCCATGAGGTCCACCAC 3'), 3 μL of RT product, and 50 units/mL Super Taq DNA polymerase. After an initial denaturation for 5 min at 95°C, 30 cycles of amplification (95°C for 1 min, 60°C for 1 min, and 72°C for 2 min) were performed, followed by a 10 min extension at 72°C[12].

V. *Flow Cytometric Detection of Cell Apoptosis*

HL-60 cells (2×10^5 /mL) were incubated with serum-free RPMI-1640 medium and treated with lovastatin at 37°C for 4 h. The apoptotic fraction was taken as the sub-G1 peak after propidium iodide (PI) staining of cells[13]. The cells were stained with 10 μg/mL propidium iodide containing 0.5% Triton X-100 and 0.1 mg/mL RNase A for 30 min. The apoptotic fraction (sub-G1 peak) was measured by flow cytometry (Becton, Dickinson and Company).

RESULTS AND DISCUSSION

Our results support the effect of lovastatin on induction of apoptosis in HL-60 cells (Figure 1). To characterize cell death induced by lovastatin, we examined the sub-G1 peak in flow cytometry with propidium iodide staining (Figure 1 upper panel), cell morphology (middle panel), and chromatin condensation with acridine orange staining (lower panel). Lovastatin exhibited significant apoptosis-inducing activity in HL-60 cells as shown in flow cytometry (with 49.83% sub-G1 peak compared to 10.43% of control), blebbing cell membrane morphology, and nuclear condensation.

While statins are highly effective in lowering serum cholesterol, they have other effects. Our data show that

342
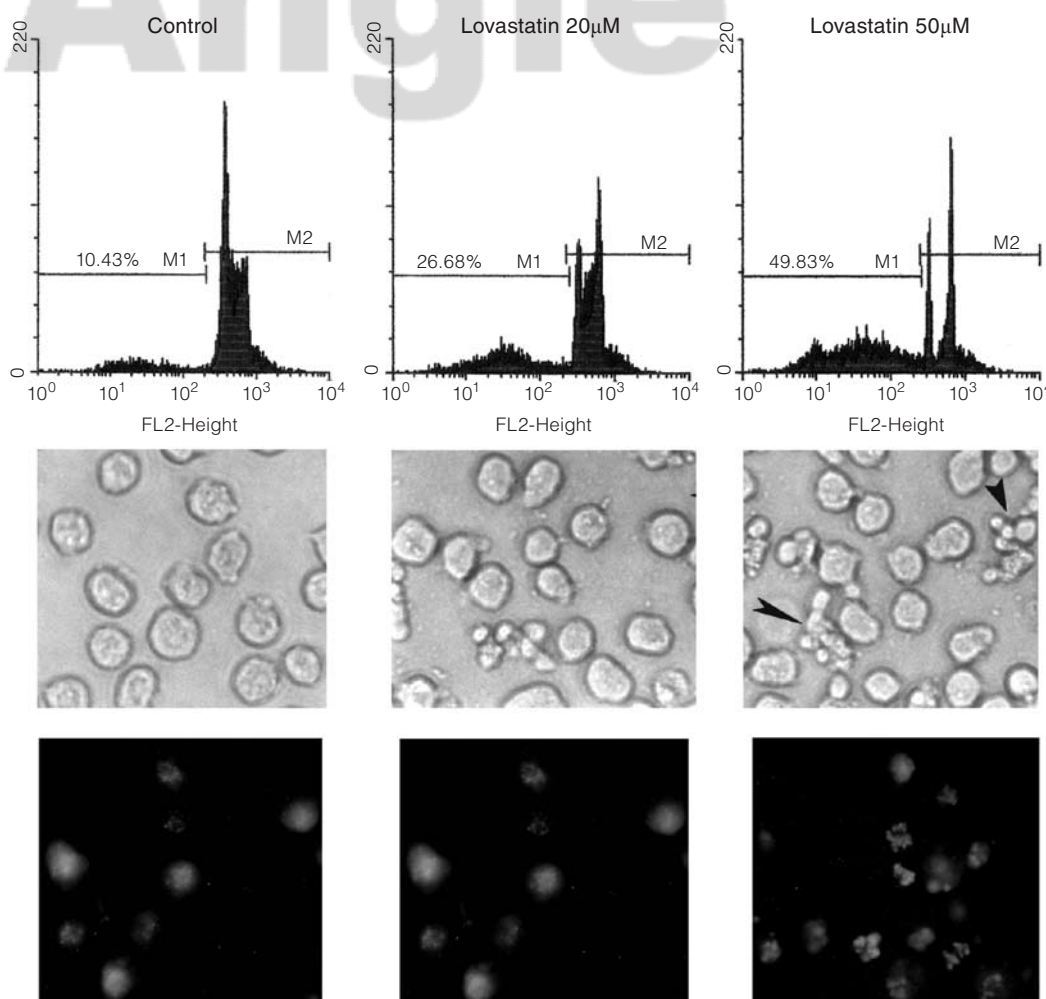
Figure 1. Lovastatin induced HL-60 cell apoptosis. Sub-G1 peak of flow cytometry by propidium iodine staining (upper panel), cell morphology (middle panel), and nuclear condensation by acridine orange staining (lower panel) were shown after lovastatin treatments (5 h).

lovastatin reduces cellular cholesterol, TG, and FFA production. The TG, cholesterol, and FFA contents of HepG2 cells were reduced to 79%, 81%, and 75% while treated with various concentrations (10, 25, and 50 μM) of lovastatin for 4 h, respectively as shown in Figure 2. FAS is normally expressed in the liver and adipose tissue, where it functions to convert dietary carbohydrate to fat. High level of synthesis of fatty acids provides proliferating cells the bulk of the endogenously synthesized fatty acids which are incorporated into membrane lipids[14]. A substantial subset of common human cancers including cancers of the prostate, breast, ovary, colon, thyroid and endometrium, have been found to express high levels of FAS[15]. Increased FAS protein expression is associated with cancer development. On the other hand, it has been shown in several studies that inhibition of FAS activity leads to cytostatic and cytotoxic effects in cultured tumor cells and significant antitumor effects in human cancers[16], thus rendering FAS a focus of anticancer drug development. Our result in Figure 3A shows that lovastatin suppresses FAS expression in HepG2 cells. Detect-
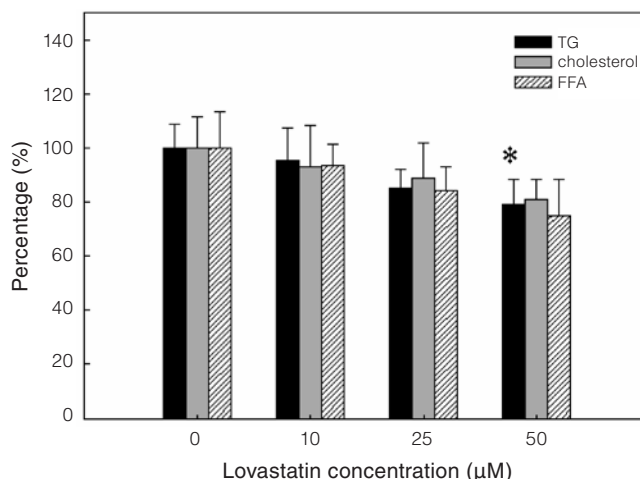
Figure 2. Lovastatin decreased cellular triglyceride, cholesterol, and free fatty acid production. The cellular triglyceride, cholesterol, and free fatty acid were decreased upon lovastatin treatments (4 hr) in HepG2 cells. Data represent means ± SD (N = 3). *p < 0.05 vs. control.

343
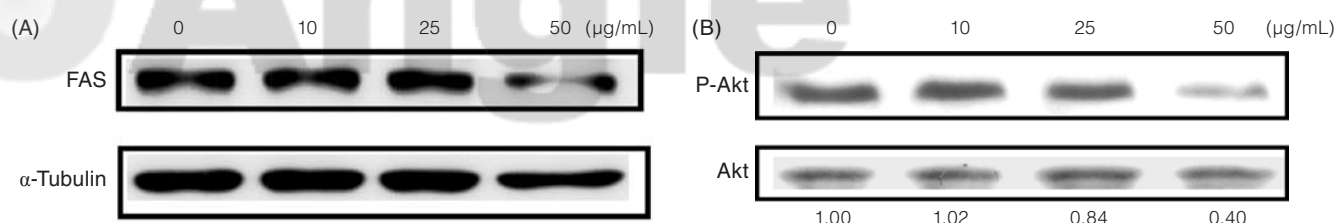
Figure 3. Lovastatin suppressed the fatty acid synthase expression and inhibited the activation of Akt in HepG2 cells. HepG2 cells were treated without (lane 1) or with lovastatin (lane 2-4) for 24 h. FAS protein levels of the cells upon various treatments were analyzed. And α-tubuline was an internal expression control (A). Phospho-Akt protein levels of the cells upon various treatments were analyzed using anti-phospho-Akt antibody in immunoblotting, and the total Akt level was an internal expression control (B). The graphs represent each experiment performed in triplicate.

able levels of FAS protein were expressed in HepG2 cells maintained under normal conditions. Suppression of FAS expression by lovastatin was detected in a concentration-dependent manner after 24 h treatment. The relative levels of FAS protein were 1.00, 0.89, 0.72, and 0.31 after treatment with 0, 10, 20, and 50 μM lovastatin, respectively. The α-tubulin with constant expression was taken as an internal control. Recent studies have revealed that FAS is upregulated through the PI3k/Akt kinase pathway to induce SREBP in cancer cells[17]. Figure 3B shows that lovastatin inhibits the activation of Akt. Given the pivotal role of Ser^{473} phosphorylation for PKB/Akt activation, the phospho-Akt (Ser^{473}) was detected by immunoblotting with anti-phospho-Akt antibody. Upon lovastatin treatment, phosphorylated Akt was reduced in a dose-dependent manner. The relative levels of phospho-Akt were 1.02, 0.84, 0.40 after treatment with 10, 20, and 50 μM lovastatin for 24 h, versus control respectively. Our results demonstrate that the Akt activation-mediating FAS overexpression is blocked by lovastatin.

To further investigate whether the suppression of FAS protein expression by lovastatin was due to the reduction of SREBP expression, a RT-PCR of total mRNA samples, extracted from HepG2 cells after 8 h treatment, was carried out. The cDNA was amplified by PCR with primers specific for SREBP-1c or GAPDH (as a control gene) and the results indicate that SREBP-1c mRNA expression is suppressed in the presence of lovastatin as shown in Figure 4. It has been reported that lovastatin induces PTEN in a dose- and time-dependent manner accompanied by a decrease in phosphorylated-AKT and phosphorylated-MAPK and an increase in G1 arrest. Lovastatin can signal through PPAR-γ and thus upregulate PTEN at the transcriptional level for therapeutic and preventative strategies[12]. This renders a linkage of decreased FAS expression and phosphorylated-AKT by lovastatin. THE RT-PCR analysis for PPAR-γ mRNA supports the effect of lovastatin on the induction of PPAR-γ in HepG2 cells (Figure 4).

Lovastatin is primarily known as an HMG-CoA reductase inhibitor which possesses a cholesterol-lowering property. Despite the inhibition of the key enzymes involved in cholesterol production, lovastatins markedly
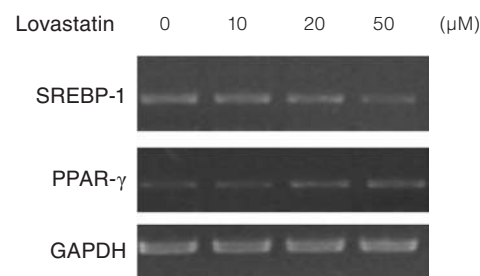
Figure 4. Lovastatin upregulated PPAR-γ and inhibited SREBP-1 mRNA expression in HepG2 cells. Total RNA of HepG2 cells treated with 0, 10, 20, 50 μM lovastatin respectively for 8h was isolated and subjected to RT-PCR with primers specific for SREBP-1 (upper) or PPAR-γ (middle). PCR-amplified cDNA was resolved in 1.2% agarose gel electrophoresis and stained with ethidium bromide. The GAPDH was an internal expression control. The graph represents an experiment performed in triplicate.

reduced TG and FFA levels (Figure 2), suggesting that different mechanisms may be involved in the hypotriglyceridemic effect of statins. Under normal physiological conditions, the increase of FAS expression in human tissues is tightly regulated by hormonal and nutritional signals[18]. In cancer cells, FAS expression has been demonstrated through phosphatidyl-inositol 3-kinase (PI3K)-Akt signaling. The inhibition of FAS is selectively cytotoxic to tumor cells and represents a cancer prevention strategy. The present study was undertaken to investigate how lovastatin is involved in the down regulation of fatty acid synthase. We found that the expression of FAS protein was reduced by lovastatin. SREBP-1 transcription was also blocked by lovastatin in a dose-dependent manner. Accordingly, we examined the effects of lovastatin on Akt phosphorylation and PPAR-γ. Our results suggest that lovastatin induces cell apoptosis and decreases cellular triglyceride, cholesterol, and FFA production by down-regulating of FAS, which is associated with increased PPAR-γ and decreased Akt activation.

Lipid rafts are cholesterol-enriched microdomains in plasma membranes. The functional activity of growth factor receptors depends on their insertion in lipid rafts which serve as membrane platforms for signaling mole-

344

cules that regulating cellular functions. Blocking of cholesterol synthesis by statin leads to lipid-raft disruption and causes cell death[19]. Cholesterol repletion replenished rafts on the cell surface and thus restored Akt activation and cell viability[20]. It has recently been suggested that activity of Fas is not only regulated by its cognate ligand but also by the association of this receptor with lipid rafts. Disruption of lipid rafts by statin causes a spontaneous clustering of Fas in the non-raft compartment of the plasma membrane, thus leading to the spontaneous, ligand-independent activation of this death receptor[21]. Our results implicate that down-regulation of FAS is associated with decreased cellular triglyceride and FFA production and might involve in the lipid-raft disruption. This study also supports a role for lovastatin as a therapeutic, adjuvant, or chemopreventive agent for certain cancers.

CONCLUSIONS

Lovastatin induces cell apoptosis and decreases cellular triglyceride, cholesterol, and FFA production by down-regulation of fatty acid synthase that is associated with decreased Akt activation and upregulating PPAR-γ.

ACKNOWLEDGEMENTS

REFERENCES

1. Ma, J., Li, Y., Ye, Q., Li, J., Hua, Y., Ju, D., Zhang, D., Cooper, R., and Chang, M. 2000. Constituents of red yeast rice, a traditional Chinese food and medicine. J. Agric. Food Chem. 48: 5220-5225.

2. Shabanzadeh, A. P., Shuaib, A., and Wang, C. X. 2005. Simvastatin reduced ischemic brain injury and perfusion deficits in an embolic model of stroke. Brain Res. 1042: 1-5.

3. Endres, M. 2005. Statins and stroke. J. Cereb. Blood Flow Metab. 25: 1093-1110.

4. Poynter, J. N., Gruber, S. B., Higgins, P. D., Almog, R., Bonner, J. D., Rennert, H. S., Low, M., Greenson, J. K., and Rennert, G.. 2005. Statins and the risk of colorectal cancer. N. Engl. J. Med. 352: 2184-2192

5. Ajith, T. A., Harikumar, K. B., Thasna, H., Sabu, M. C., and Babitha, N. V. 2006. Proapoptotic and antitumor activities of the HMG-CoA reductase inhibitor, lovastatin, against Dalton's lymphoma ascites tumor in mice. Clin. Chim. Acta. 366: 322-328.

6. van de Donk, N. W., Bloem, A. C., van der Spek, E., and Lokhorst, H. M. 2006. New treatment strategies for multiple myeloma by targeting BCL-2 and the mevalonate pathway. Curr. Pharm. Des. 12: 327-340.

7. Yang, Y. A., Han, W. F., Morin, P.J., Chrest, F.J., and Pizer, E. S. 2002. Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Exp Cell Res. 279: 80-90.

8. Stoeckman, A. K., and Towle, H.C. 2002. The role of SREBP-1c in nutritional regulation of lipogenic enzyme gene expression. J. Biol. Chem. 277: 27029-27035.

9. Schuster, G. S., Erbland, J.F., Lefebvre, C. A., Caughman, G. B., and Knoernschild, K. L. 1997. Effects of an aminomethacrylate on epithelial cell lipid metabolism. J. Biomater. Sci. Polym. Ed. 8: 363-375.

10. Patterson, B. W., Zhao, G., Elias, N., Hachey, D. L., and Klein, S. 1999. Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment. J. Lipid Res. 40: 2118-2124.

11. Lin, C. M., Huang, S. T., Liang, Y. C., Lin, M. S., Shih, C. M., Chang, Y. C., Chen, T. Y., and Chen, C. T. 2005. Isovitexin suppresses lipopolysaccharide-mediated inducible nitric oxide synthase through inhibition of NF-kappa B in mouse macrophages. Planta Med. 71: 748-753.

12. Teresi, R. E., Shaiu, C. W., Chen, C. S., Chatterjee, V. K., Waite, K. A., and Eng, C. 2006. Increased PTEN expression due to transcriptional activation of PPARgamma by Lovastatin and Rosiglitazone. Int. J. Cancer. 118: 2390-2398.

13. Lin, C. M., Chen, C. T., Lee, H. H., and Lin, J. K. 2002. Prevention of cellular ROS damage by isovitexin and related flavonoids. Planta Med. 68: 365-367.

14. Swinnen, J. V., Van Veldhoven, P. P., Timmermans, L., De Schrijver, E., Brusselmans, K., Vanderhoydonc, F., Van de Sande, T., Heemers, H., Heyns, W., and Verhoeven, G. 2003. Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochem. Biophys. Res. Commun. 302: 898-903.

15. Swinnen, J. V., Roskams, T., Joniau, S., Van Poppel, H., Oyen, R., Baert, L., Heyns, W., and Verhoeven, G. 2002. Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. Int. J. Cancer. 98: 19-22.

16. Kuhajda, F. P. 2006. Fatty acid synthase and cancer: new application of an old pathway. Cancer Res. 66: 5977-5980.

17. Chang, Y., Wang, J., Lu, X., Thewke, D. P., and Mason, R. J. 2005. KGF induces lipogenic genes through a PI3K and JNK/SREBP-1 pathway in H292 cells. J. Lipid Res. 46: 2624-2635.

18. Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T., and Tanaka, T. 1990. Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of fatty acid synthase in rat liver. Eur. J. Biochem. 1902: 427-433

19. Bang, B., Gniadecki, R., and Gajkowska, B. 2005. Disruption of lipid rafts causes apoptotic cell death in

345

HaCaT keratinocytes. Exp. Dermatol. 14: 266-272.

20. Li, Y. C., Park, M. J., Ye, S. K., Kim, C. W., and Kim, Y. N. 2006. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. Am. J. Pathol. 168: 1107-1118

21. Gniadecki, R. 2004. Depletion of membrane cholesterol causes ligand-independent activation of Fas and apoptosis. Biochem. Biophys. Res. Commun. 320:165-169.