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ORIGINAL ARTICLE

Suppression of fatty acid synthase in MCF-7 breast cancer cells by tea and tea polyphenols: a possible mechanism for their hypolipidemic effects

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ABSTRACT

Tea is a heavily consumed beverage world wide because of its unique aroma, less cost and broad availability. Fatty acid synthase (FAS) is a key enzyme in lipogenesis. FAS is overexpressed in the malignant human breast carcinoma MCF-7 cells and its expression is further enhanced by the epidermal growth factor (EGF). The EGF-induced expression of FAS was inhibited by green and black tea extracts. The expression of FAS was also suppressed by the tea polyphenol (−)-epigallocatechin 3-gallate (EGCG), theaflavin (TF-1), TF-2 and theaflavin 3,3′-digallate(TF-3) at both protein and mRNA levels that may lead to the inhibition of cell lipogenesis and proliferation. Both EGCG and TF-3 inhibit the activation of Akt and block the binding of Sp-1 to its target site. Furthermore, the EGF-induced biosyntheses of lipids and cell proliferation were significantly suppressed by EGCG and TF-3. These findings suggest that tea polyphenols suppress FAS expression by downregulating EGF receptor/PI3K/Akt/Sp-1 signal transduction pathway, and tea and tea polyphenols might induce hypolipidemic and antiproliferative effects by suppressing FAS. *The Pharmacogenomics Journal* (2003) **3**, 267–276, doi:10.1038/sj.tpj.6500192; Published online 19 August 2003

Keywords: FAS; EGF; MCF-7 breast cancer cell; tea polyphenols; lipogenesis; proliferation; PI3K/Akt/Sp-1 signalling

INTRODUCTION

It is obvious that excess body weight is a major health problem in most developed nations and is increasing both in prevalence and severity.[1] As obesity is a major risk factor for cardiovascular disease, diabetes and cancer, for which the social costs are incalculable, it is regarded as one of the most dangerous noninfectious threats to health worldwide. Development of drugs to treat obesity or implementation of a dietary regimen to prevent obesity is a public health goal of the highest priority.

Breakthroughs in our understanding of the molecular mechanisms regulating body weight have provided potential opportunities for therapeutic intervention and brought renewed hope and vitality in the development of antiobesity drugs. In the last decade, several candidate drugs have been considered as treatments for obesity. The hormone leptin causes profound obesity when it is absent.[2] Leptin appears to act primarily by regulating the activity of specific hypothalamic neurons. Unfortunately, leptin fails to reduce body weight in most forms of obesity, presumably because of the development of leptin resistance.

Fatty acid synthase (FAS) is an important enzyme participating in energy metabolism[3] and is related to various human diseases including obesity, cardiovascular disease and cancer. Human cancer cells express high levels of FAS and inhibition of FAS is selectively cytotoxic to human cancer cells.[4] Inhibitors of FAS such as cerulenin and C75 are known for their effects on some human cancer.[5]

FAS inhibitors produce metabolic effects similar to those produced by leptin, apparently by increasing malonyl-CoA levels in nutrition-sensitive hypothalamic neurons.[6] However, acute treatment with C75 or its progenitor, cerulenin robustly reduces body weight in forms of obesity that are resistant to leptin.[7,8] Regardless of the mechanism, FAS inhibitors clearly activate potent weight-reducing pathways, so compounds that regulate convergent hypothalamic pathways and/or novel neuropeptide systems activated by FAS inhibitors might be promising candidates for antiobesity drugs.

Obesity is a chronic, stigmatized and costly disease that is rarely curable. Presently, available treatments including drugs are palliative and are effective only while the treatment is being effectively used.[9] Any effective drug will be widely used for its cosmetic as well as medical benefits. Thus, any drug that is approved should meet high standards for safety.

While many investigators are looking for a magic drug to combat obesity, some researchers are searching for an effective daily dietary remedy in preventing surplus weight gain. It seems that obesity is rare in the vegetarian population, but most vegetarian foods are not attractive to the general population. It is worth noting that tea-drinking is a very popular habit in the Oriental population, especially in China and Japan.

Both black tea and green tea are widely consumed beverages and contain biologically active tea polyphenols (Figure 1). Green tea contains six catechins, namely (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), (−)-epigallocatechin ((EGC), (−)-epigallocatechin 3-gallate (EGCG) and (−)-gallocatechin 3-gallate (GCG). It seems that EGCG is the most abundant and biologically active catechin, while GCG is the artificial isomer of EGCG produced from chemical handling. In the process of tea manufacturing and fermentation, the black tea polyphenols theaflavins are formed by oxidative dimerization of catechins. Theaflavins are composed of theaflavin (TF-1), theaflavin 3-gallate (TF-2a), theaflavin 3′-gallate (TF-2b) and theaflavin 3,3′-digallate(TF-3). After testing in several biological systems, TF-3 was found to be the most biologically active theaflavin.

Based on a general observation, obesity is quite rare in the long-term tea-drinking inhabitants. This striking phenomenon encouraged us to carry out animal experiments to investigate the hypolipidemic and antiobesity effects of tea. The results of our animal experiments are quite encouraging.[10] Male Wistar rats were fed 2.5% pulverized green tea (Chinese Longjing tea) leaves for 63 weeks. The changes of GOT, GPT, γGT and creatinine were not significant in the treated group as compared with the control. These results

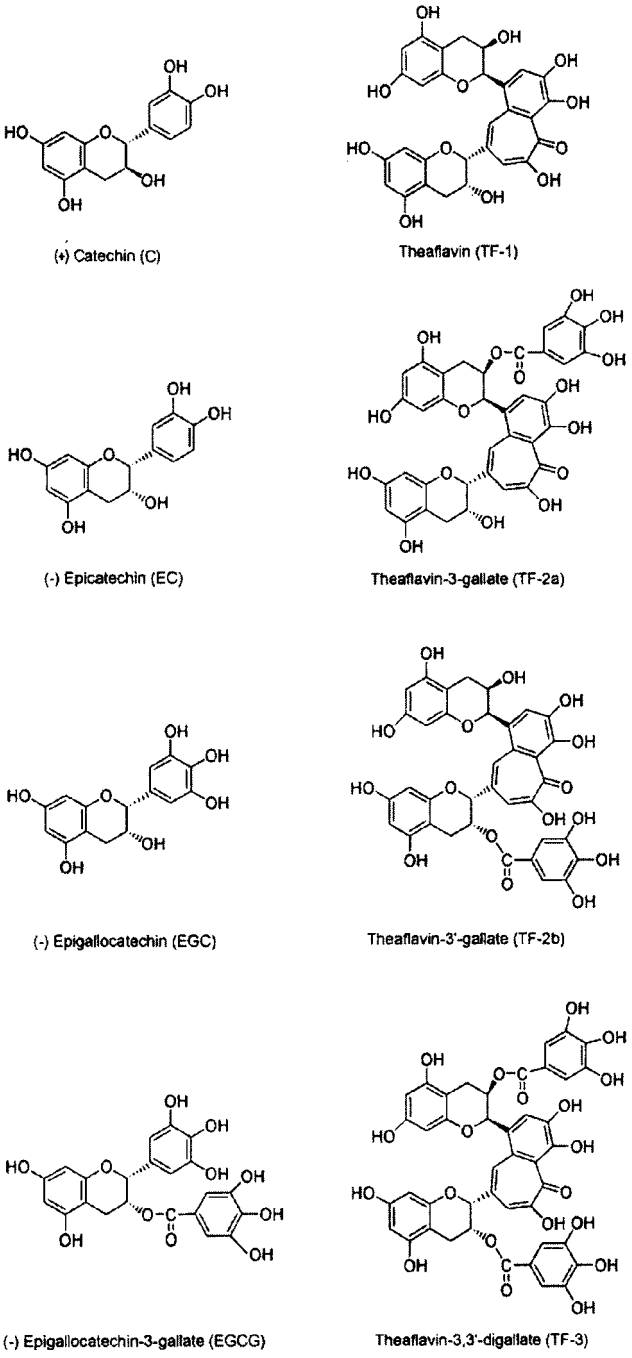

Figure 1 Chemical structures of tea polyphenols. Green tea polyphenols: catechin, epicatechin, epigallocatechin and epigallo - catechin 3-gallate; Black tea polyphenols: theaflavin, theaflavin 3-gallate: theaflavin 3′-gallate and theaflavin 3,3′-digallate.

suggested that long-term feeding of green tea leaves was not toxic to the liver or kidney. Very significant body weight reduction and hypolipidemic effect of green tea leaves were observed in the treated rats. Serum total cholesterol, triglyceride, and LDL-cholesterol levels were decreased in the tested group. The dietary intakes of the two groups were approximately the same, but the body weights of the tea-fed

group were decreased 10–18% compared with those of the control group.[10]

The activities of antioxidant enzymes such as superoxide dismutase and catalase, and phase II enzymes such as glutathione *S*-transferase and glutathione concentration in the liver of Wistar rats were significantly higher in the treated group.[10,11] The biological significance of these results can be implicated in relation to the hypolipidemic effect as well as cancer chemopreventive action of green tea. Similar studies by Yang and Koo[12] have shown that Chinese jasmine green tea has significant serum- and liver-cholesterol lowering effects. Kao *et al*[13] reported that injection of the tea polyphenol EGCG into mice reduced their food intake and body weight, but the mechanism remains to be elucidated. Recently, an *in vitro* study has shown that EGCG is an effective inhibitor of FAS isolated from chicken liver.[13]

It seems that because most previous studies on the FAS inhibitors have been confined to their inhibitory effects on the enzyme activity of FAS,[5,7,8,14] it would be more interesting to investigate the inhibitory effects of these compounds on the enzyme biosynthesis of FAS. Our previous studies[10] showed that long-term feeding of Chinese Longjing green tea leaves to rats could reduce their body weight and lipid contents without reducing food intake. This indicated that the input of exogenous lipid had been kept the same, but the biosynthesis of endogenous lipid could be suppressed, or on the other hand, the oxidation of cellular lipid could be accelerated. Our working hypothesis is that tea or tea polyphenols may exert their hypolipidemic and antiobesity actions by suppressing the biosynthesis of FAS. In order to substantiate this hypothesis, we have further performed a series of experiments in human MCF-7 breast cancer cells to demonstrate the suppression of FAS protein and mRNA expression by tea extracts and tea polyphenols, including catechins such as C, EC, EGC and EGCG and theaflavins including TF-1, TF-2 and TF-3. Furthermore, the molecular mechanism of this suppression is also studied.

EXPRESSION OF FAS IS HIGHER IN CANCEROUS CELLS THAN IN IMMORTALIZED CELLS

A considerably high expression of FAS is observed in cancerous tissues than in normal tissues. In order to demonstrate the high expression of FAS in cancer cells, the FAS protein level in human breast cancer cell line MCF-7 and immortalized breast epithelium cells HBL-100 was assayed (Figure 2a). In contrast to HBL-100 cells, a rather high level of FAS was observed in MCF-7 cells. The FAS protein in MCF-7 cells is about 2.5-fold higher than that in HBL-100 cells. This confirmed that the FAS protein level is higher in cancerous cells. Owing to the high expression of FAS, we chose MCF-7 cells as a model for the following studies.

Black and Green Tea Extracts, But Not Oolong Tea Extracts, Inhibit FAS Protein

Suppression of FAS in cancer cells may lead to growth inhibition and cell apoptosis.[15] Therefore, we examined whether extracts from different tea have effects on down-regulation of FAS protein in MCF-7 human breast cancer cell line. Cells were cultured in DMEM with 5% fetal calf serum and treated with tea extracts from oolong, black and green tea to examine their effects on the expression of FAS. As shown in Figure 2b, tea extracts from oolong, black and green tea seemed to stimulate the expression of FAS at a lower concentration (30 μg/ml). However, when cells were exposed to a higher concentration of tea extracts (120 μg/ml), the expression of FAS was inhibited by green and black tea extracts, but not by the oolong tea extract. This

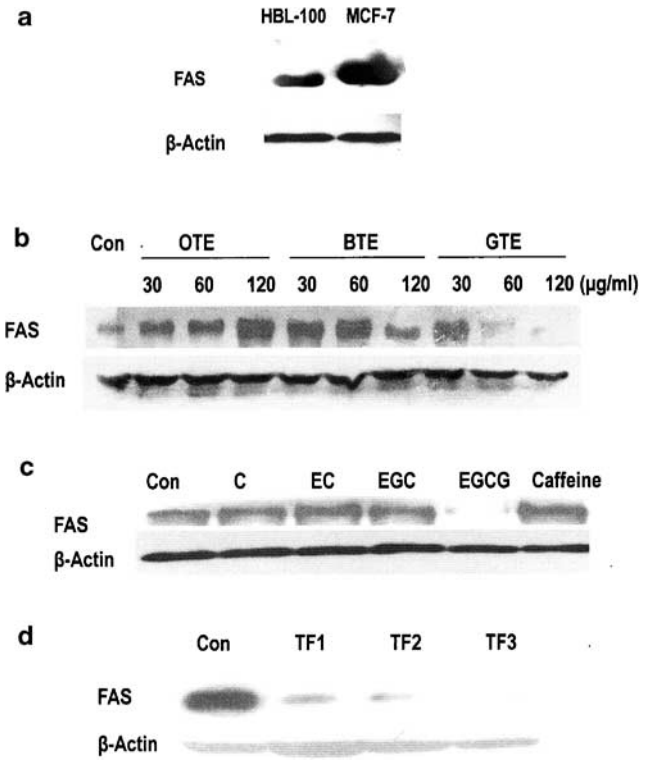

Figure 2 Green tea and black tea extracts suppress FAS expression in MCF-7 cells. (a) Expression of FAS in HBL-100 and MCF-7 cells. Cells were cultured in DMEM with 10% FCS. After attachment for 18 h, HBL-100 and MCF-7 cells were harvested. Lysis of cells and Western blotting were performed as described in 'Methods'. β-Actin was the internal control. (b) Effect of different teas on FAS expression in MCF-7 cells. MCF-7 cells were cultured in DMEM with 5% FCS and treated with different tea extracts at indicated doses. After incubation for 24 h, cells were harvested. Con, control. OTE, oolong tea extracts. BTE, black tea extracts. GTE, green tea extracts. (c) MCF-7 cells were cultured in DMEM with 5% FCS. Green tea catechins and caffeine were added to the medium at 30 μM. After incubation for 24 h, cells were harvested. Abbreviations are: Con, control; C, catechin; EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate. (d) MCF-7 cells were cultured in DMEM with 5% FCS. Black tea theaflavins were added to the medium at 30 μM. After incubation for 24 h, cells were harvested. Abbreviations are: Con, control; TF-1, theaflavin; TF-2, a mixture of theaflavin-3-gallate and theaflavin-3′-gallate; TF-3, theaflavin-3, 3′-digallate.

suggested that effects of various components of tea extract on FAS expression might be different.

EGCG and TF-3 Inhibit FAS Protein

It has been demonstrated that most biological and pharmacological activities of tea are attributed to its tea polyphenols.[16,17] To elucidate the effects of tea polyphenols on FAS, various green tea catechins, black tea theaflavins and caffeine were examined to determine whether they affect the expression of FAS in MCF-7 cells (Figure 2c and d). In green tea catechins, only EGCG was found to reduce the amount of FAS protein significantly by 76% at a dose of 30 μM. Catechin (C), epicatechin (EC), epigallocatechin (EGC) and caffeine had slight enhanced effects on FAS protein expression (Figure 2c). To our surprise, the black tea polyphenols TF-1, TF-2 and TF-3 actively downregulated FAS by 52, 69 and 87% at 30 μM, respectively (Figure 2d). TF-3 was the most active in reducing the FAS protein.

EGCG and TF-3 Inhibit EGF-induced Expression of FAS Protein and mRNA

Recent studies have shown that many sex hormones and growth factors in serum could upregulate FAS in cancers.[18] Epidermal growth factor (EGF) is a candidate of those cytokines. After serum starvation for 24 h, MCF-7 cells were treated with EGF (100 ng/ml); a two-fold increase in FAS protein induction was observed (Figure 3a). EGCG could suppress this FAS induction by 49, 60 and 62% at 10, 20 and 40 μM, respectively. Similar to the effects of EGCG, TF-3 also downregulated FAS by 31, 54, 87 and 100% at 5, 10, 20 and 40 μM, respectively. Decrease in the level of FAS protein may be caused by reduced FAS mRNA expression or instable FAS protein. To elucidate the exact mechanism that affects the levels of FAS expression, MCF-7 cells treated with EGF without or with various doses of EGCG and TF-3 for 12 h were harvested and assayed for FAS mRNA by reverse transcription-polymerase chain reaction (RT-PCR) (Figure 3b). FAS mRNA was present as a single band of ~250 bp. The data in Figure 3b demonstrate upregulation of FAS mRNA levels after induction with EGF. In the presence of EGCG or TF-3, FAS mRNA decreased in a dose-dependent manner. The magnitude of decreased FAS mRNA for EGCG is 44, 69 and 99% at 10, 20 and 40 μM, respectively. TF-3 also had the same effect on the downregulation of FAS mRNA. At 5, 10 and 20 μM, TF-3 inhibited the level of FAS mRNA by 15, 40 and 57%. The present data suggest that EGCG and TF-3 inhibit the expression of FAS through transcriptional regulation.

The suppression of EGF-induced FAS protein by EGCG and TF-3 was also demonstrated in human hepatoblastoma HepG2 cells (Figure 3). It appeared that the expression of FAS in the Hep G2 cells was more tightly regulated by insulin (Figure 3d) than by EGF (Figure 3c). Under the same experimental conditions, the insulin-enhanced FAS expression was significantly suppressed by EGCG and TF-3 (Figure 3d), while the EGF was unable to enhance the FAS expression, but the basal level of FAS in the treated cells could be suppressed by the tea polyphenols (Figure 3c).

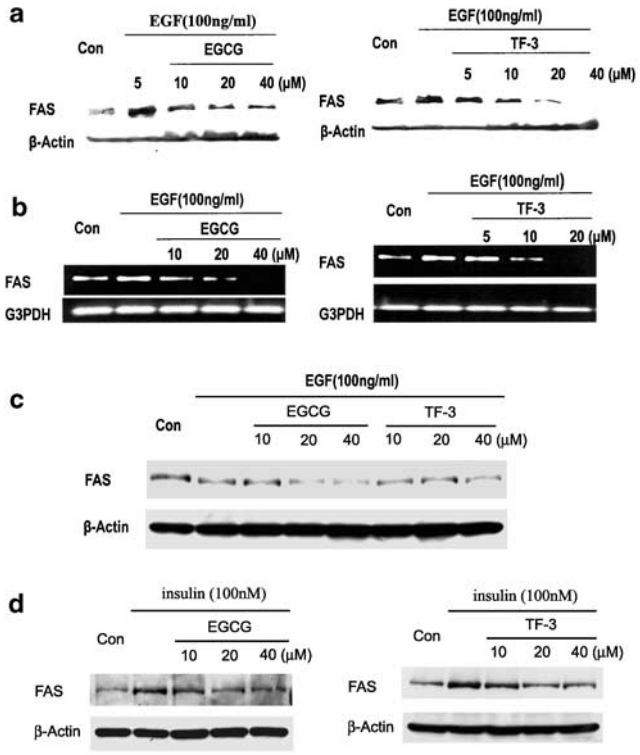

Figure 3 EGCG and TF-3 reduce the expression of FAS protein and mRNA in MCF-7 and HepG2 cells. (a) Western blotting analysis of total cell lysate from MCF-7 cells. MCF-7 cells were pretreated with various indicated doses of EGCG and TF-3 for 30 min before EGF (100 ng/ml) treatment. After incubation for 24 h, cells were harvested and the expression of FAS protein was analyzed. (b) RT-PCR analysis of cDNA from MCF-7 cells. Cells were pretreated with EGCG and TF-3 at indicated doses for 30 min before EGF(100 ng/ml) treatment. After incubation for 12 h, total RNA was isolated and the RNA expression was analyzed by RT-PCR as described in 'Methods'. (c) Western blotting analysis of total lysate from EGF-induced HepG2 cells. HepG2 cells were pretreated with various indicated doses of EGCG and TF-3 for 30 min before EGF (100 ng/ml) treatment. After incubation for 24 h, cells were harvested and the expression of the FAS protein was analyzed. (d) Western blotting analysis of total lysate from insulin-induced HepG2 cells. HepG2 cells were pretreated with various doses of EGCG and TF-3 for 30 min before insulin (100 nM) treatment. After incubation for 24 h, cells were harvested and the expression of the FAS protein was analyzed.

Inhibitor of Phosphatidylinositol-3-kinase, Ly294002, Inhibits Expression of FAS in MCF-7 Cells Stimulated by EGF

Phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway has been demonstrated to upregulate the expression of FAS in LNCaP human prostate cancer cell line.[19] We have examined whether the same pathway was involved in the induction of the FAS protein in MCF-7 cells. We treated cells with the inhibitor of PI3K, Ly294002 at 1, 5, and 10 μM. The results showed that Ly294002 nearly completely abolished the effects of EGF on FAS expression at 5 μM (Figure 4a). Furthermore, the induced FAS protein was significantly suppressed by transient transfection of

dominant-negative Akt mutant gene into the MCF-7 cells (Figure 4b). These results suggested that PI3K/Akt signal transduction pathways might be involved in activating the expression of FAS and any blockage of the pathway would reduce the amount of FAS in MCF-7 cells.

EGCG and TF-3 Block Activation of Akt Kinase but not Mitogen-activated Protein Kinase

As demonstrated in the previous experiment, the PI3K/Akt signal transduction pathway may be involved in EGF induction of FAS in MCF-7 cells, so we explore the effect of EGCG and TF-3 on the PI3K/Akt pathway (Figure 4c). The data demonstrated that EGF (100 ng/ml) activated Akt kinase. 10 and 20 μM EGCG had no or a slightly inhibitory effect on the phosphorylation of Akt kinase, but at 40 μM concentration, EGCG would reduce phosphorylation of Akt by 52%. TF-3 had a similar dose-dependent inhibitory effect on the activation of Akt kinase. It inhibited phosphorylation of Akt kinase by 72% at 20 μM (Figure 4d). In the p44/p42 MAPK signal transduction pathway, EGF also enhanced the activation of ERK (Figure 4c). However, at the same concentration EGCG-blocking Akt activation had no inhibitory effects on phosphorylation of MAP kinase. ERK seemed to be activated by EGCG (Figure 4c). On the other hand, TF-3 could slightly inhibit phosphorylation of ERK at 20 μM (Figure 4d). These results indicated that EGCG and TF-3 showed different effects on the activation of p44/p42 MAPK signal tranduction pathway.

Binding Capacity of Sp-1 was Suppressed by EGCG and TF-3

The transcription factor Sp-1, a member of the closely related family of proteins that includes Sp-2, Sp-3 and Sp-4, is ubiquitously expressed and binds to GC-rich sequences ('GC boxes') with the consensus sequence 5′-G/TG/AGGCG/AG/AG/T-3′. Studies showed that Sp-1, together with sterol-responsive element-binding protein-1 (SREBP-1) or SREBP-2, is essential in the sterol regulation of the rat FAS gene. Analyzed by Western blot assay, EGCG and TF-3 did not reduce Sp-1 protein (data not shown). Electrophoresis mobility shift assay was used to examine whether the binding capacity of Sp-1 was influenced by EGCG and TF-3. As shown in Figure 4e, the data indicated that both compounds inhibited EGF-stimulated Sp-1 binding activity. As the DNA binding capacity of Sp-1 could be regulated by phosphorylation, EGCG and TF-3 might influence the phosphorylation of Sp-1 and lead to protein conformation change. EGCG inhibited DNA binding of Sp-1 in a dose-

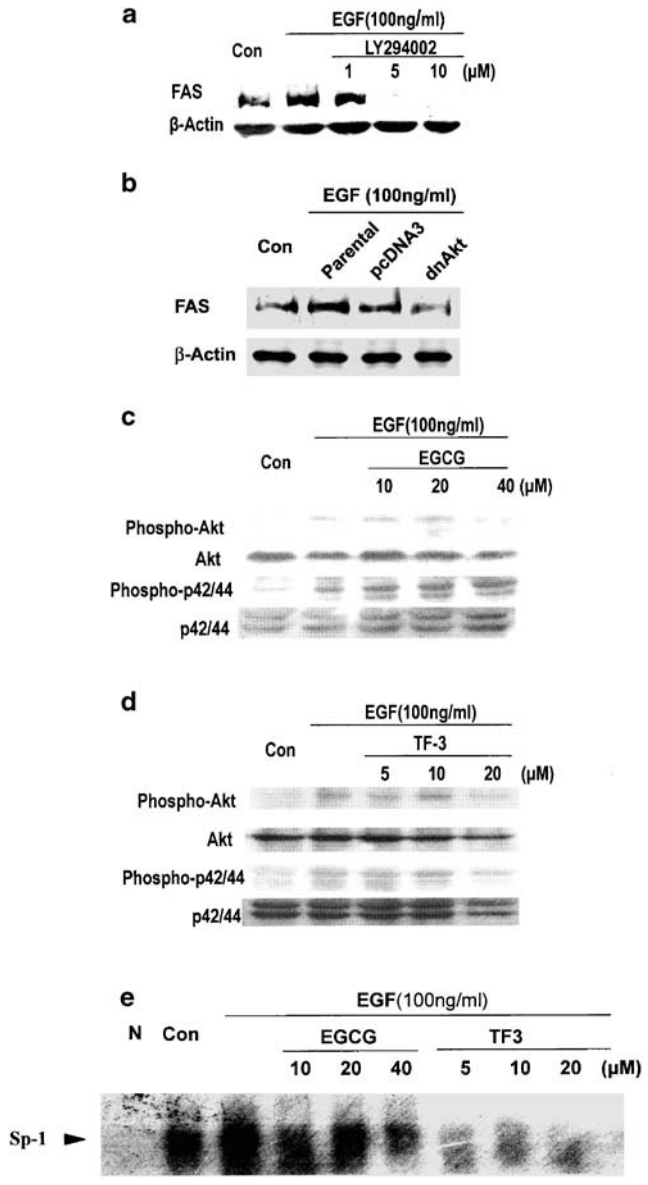

Figure 4 PI3K/Akt signal pathway is necessary for the expression of FAS. (a) Western blotting analysis of total cell lysate from MCF-7 cells. Cells were pretreated with Ly294002 at indicated doses for 2 h before EGF treatment. After incubation for 24 h, cells were harvested and the expression of the FAS protein was analyzed. (b) Dominant-negative Akt inhibits EGF-regulated FAS expression. MCF-7 cells were transfected with dnAkt (kinase-defective Akt, Akt K179A) or control vector (pcDNA3) and subsequently treated with EGF (100 ng/ml) for 24 h. Cell lysates were analyzed by Western blotting using anti-FAS antibody. β-Actin was used as a loading control. (c) Effect of EGCG on the phosphorylation of Akt kinase and p44/42 ERK. Cells were treated with EGCG at indicated doses for 30 min before EGF (100 ng/ml) treatment. After incubation for 15 min, cells were harvested and total cell lysate was analyzed. (d) Effect of TF-3 on the phosphorylation of AKt kinase and p44/42 ERK. Cells were treated with TF-3 at indicated doses for 30 min before EGF (100 ng/ml) treatment. After incubation for 15 min, cells were harvested and total cell lysate was analyzed. (e) Effects of EGCG and TF-3 on the binding capacity of Sp-1. Electrophoresis mobility shift assay was used to analyze the DNA binding capacity of Sp-1. EGF, EGCG and TF-3 were treated at indicated doses. After incubation for 12 h, cells were harvested and analyzed as described in 'Methods'. The triangle indicates the shift bands. N, labeled DNA alone; Con, control.

dependent manner at the concentration range of 10–40 μM. Meanwhile, TF-3 greatly inhibited the DNA binding of Sp-1 at 5 μM. These results might indicate that both EGCG and TF-3 have different affinity to the Sp-1 binding protein.

Lipid Biosynthesis in MCF-7 Cells was Suppressed by EGCG and TF-3

In order to examine whether lipid biosynthesis could be blocked by EGCG and TF-3, U-^{14}C acetyl-CoA was added to the cultivating MCF-7 cells with or without EGF and isotope-labeled lipids were extracted and analyzed as described in the Methods. The basal levels of lipid biosyntheses in control cells without EGF were set as 100% (Figure 5a and b). The growth factor EGF (100 ng/ml) enhanced the biosyntheses of different lipids, including cholesterol ester (116%), triglyceride (123%), free fatty acid (194%) and free cholesterol (207%). The EGF-induced biosyntheses of free fatty acid and free cholesterol were significantly inhibited by EGCG (10 μM) and down to 106 and 103%, respectively. The inhibitions were declined slightly (132 and 148%, respectively) when the concentration of EGCG was elevated to 20 μM. Meanwhile, the EGF-induced biosyntheses of free fatty acid and free cholesterol were significantly inhibited by TF-3 (10 μM) and down to 92 and 93%, respectively. The inhibitions were dose-dependent and reached 82 and 80%, respectively, when the concentration of TF-3 was elevated to 20 μM (Figure 5b). The EGF-induced biosynthesis of triglyceride were suppressed by EGCG (10 μM, 77%; 20 μM, 96%) and TF-3 (10 μM, 70%; 20 μM, 83%), respectively. The EGF-induced biosynthesis of cholesterol ester was also suppressed by EGCG to a lesser extent (10 μM, 100%; 20 μM, 114%) and by TF-3 to a greater extent (10 μM, 91%; 20 μM, 56%), respectively (Figure 5b). It is apparent that the black tea polyphenol TF-3 inhibited the various lipid biosyntheses more prominently while the green tea polyphenol EGCG showed a lower inhibitory effect.

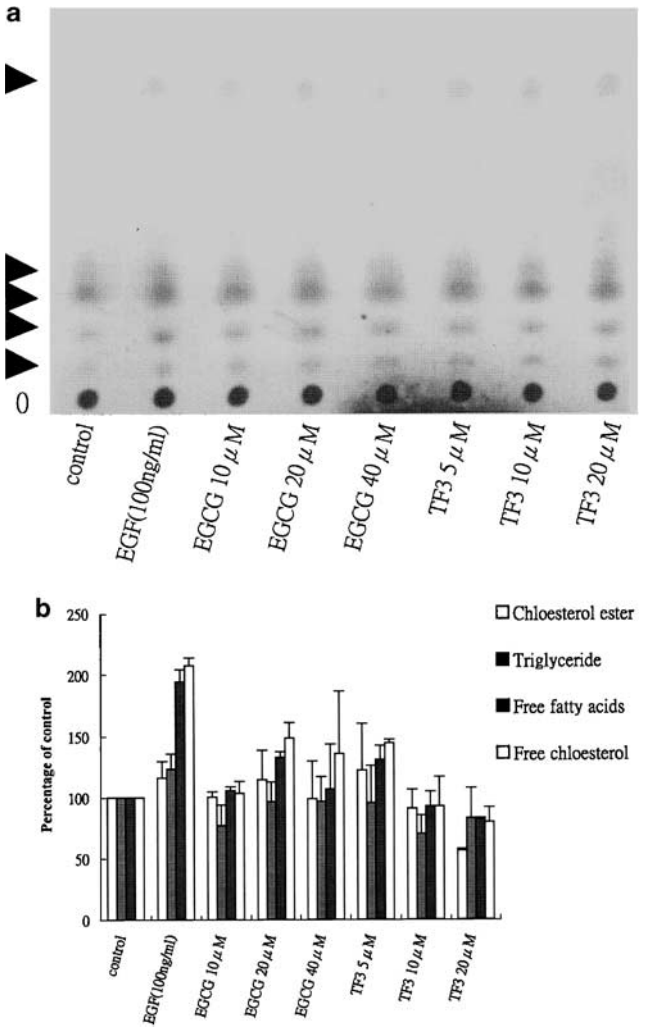

Figure 5 EGCG and TF-3 can reduce lipid biosynthesis in MCF-7 cells. (a) Autoradiography of the TLC sheet. MCF-7 cells were treated with EGCG and TF-3 at indicated doses for 30 min before EGF (100 ng/ml) treatment. After 24 h, cells were incubated for 2 h with U-^{14}C acetyl-CoA. Total lipids were extracted and separated as described in 'Methods'. Cholesterol ester, triglyceride, free fatty acids, free cholesterol and phospholipids were separated from top to bottom on a TLC sheet and indicated with black triangles. (b) Levels of cholesterol, cholesterol ester, free fatty acids and triglyceride were presented as mean ± SE from three different experiments.

DISCUSSION

The expression of FAS was upregulated by EGF as demonstrated by elevated levels of protein and mRNA (Figure 3a and b). The expression of EGF-induced FAS was significantly inhibited by EGCG and TF-3 (Figure 3a and b). There are two possible downstream signal pathways of EGF receptor leading to FAS transcription, such as MAPK/ERK/ELK and PI3k/AKt/SP-1 pathways. Our present results suggest that EGF may bind to its receptor and induce the expression of FAS through PI3k/AKt/Sp-1 pathways (Figure 4). In this signal transduction pathway, EGF receptor is the primary regulatory site for EGF ligand binding that induces the autophosphorylation of the EGF receptor at tyrosine residue in its cytoplasmic domain. The phosphorylated EGF receptor subsequently transfers its signals to downstream transducer proteins such as Grb-2, SOS, Ras, Raf-1, MAPKs or PI3K/Akt. In 1997, we demonstrated that tea polyphenols including EGCG and TF-3 could profoundly inhibit the binding of EGF to its receptor and then suppress the autophosphorylation of EGF receptor.[20] Therefore, it is quite likely that inhibition of tea polyphenols on EGF receptor activation may be the first crucial step for their suppression on FAS gene expression.

The important biological function of FAS in cancer development has been recognized for long time.[21] The majority of human cancers, including cancer of the breast, colon, ovary, lung and prostate express elevated levels of FAS.[4] Inhibition of FAS activity inhibits tumor cell growth and induces apoptosis[15] rendering FAS an important target for anticancer drug development.[5] Factors that have shown to play an important role in the control of lipogenic enzyme

in cancer cells are growth factors such as EGF, of which the signaling pathways are frequently dysregulated and constitutively activated in cancer cells.[22] One of the intracellular signaling pathways that are frequently activated in cancer cells is the PI3K/Akt kinase pathway.[23] This pathway has been documented as an important signaling for cell survival, cell transformation and tumor growth. PI3K catalyzes the formation of the 3′ phosphoinositides, phosphatidylinositol 3,4-diphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate. Increases in 3′ phosphoinositides lead to membrane translocation of downstream effectors such as the serine/threonine protein kinase Akt. On translocation, Akt is phosphorylated and activated by phosphatidylinositol 3,4,5-triphosphate-dependent kinase (PDK), ultimately resulting in the stimulation of cell growth and survival through transcription activation of the FAS gene (Figure 6). Our present data indicate that the activated Akt may bind on the SP-1 site that leads to transcription of the FAS gene (Figure 4e). As part of our studies to elucidate the molecular mechanisms underlying the suppression of FAS in cancer cells, we have used MCF-7 human breast cancer cells as the paradigm of the FAS-overexpression system and demonstrated the suppression of FAS expression by tea polyphenols including EGCG, TF-1, TF-2 and TF-3.

FAS plays a central role in *de novo* lipogenesis in mammals.[24] By the action of its seven active sites, FAS catalyzes all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS concentration is sensitive to nutritional and hormonal status in lipogenic tissues such as liver and adipose tissue, being undetectable in the livers of starved mice and dramatically induced upon refeeding a high carbohydrate, fat-free diet.[25] The nutritional regulation of FAS occurs mainly via changes in FAS gene transcription.[26] The present study showed that the incorporation of ^{14}C-acetyl-CoA into cholesterol, fatty acid and triglyceride in MCF-7 cells was significantly inhibited by EGCG and TF-3 (Figure 5). These results have provided evidence that tea polyphenols such as EGCG and TF-3 profoundly suppress FAS gene transcription through EGF/PI3K/Akt/Sp-1 signal pathways as illustrated in Figure 6.

The cancer chemopreventive effects of tea and tea polyphenols have been intensively investigated.[16,17,27] The cancer chemopreventive action of tea has been attributed to its major phytopolyphenols. The tea polyphenols comprise about one-third of the weight of the dried leaf, and they show profound biochemical activities including antioxidant activities,[28] modulation of carcinogen metabolism, inhibition of cell proliferation,[20] induction of cell apoptosis[29] and cell cycle arrest.[30] They intervene in the biochemical and molecular processes of multistep carcinogenesis, comprising tumor initiation, promotion and progression. Most tea polyphenols exert their scavenging effects against reactive oxygen species (ROS);[28] excessive production of ROS has been implicated for the development of cardiovascular disease, neurodegenerative disorders and cancer. Recently, we have found that EGCG and TF-3 suppress extracellular signals and cell proliferation through EGF receptor activation in human A431 epidermoid carcinoma cells.[20] EGCG

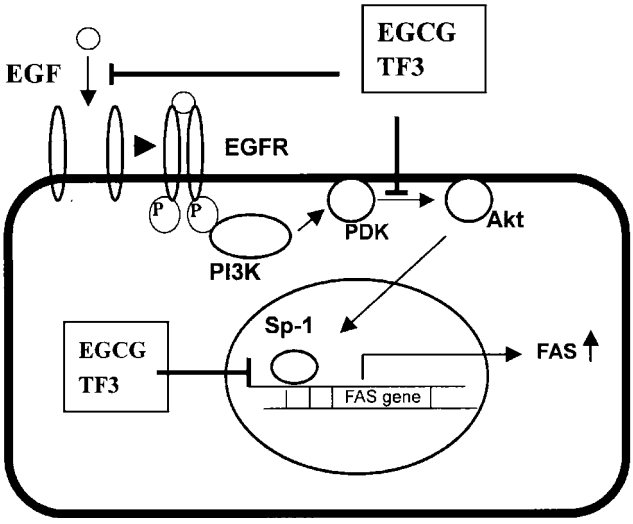

Figure 6 A proposed mechanism for the suppression of EGCG and TF-3 on the expression of FAS through modulation of the EGF receptor/PI3K/Akt/Sp-1 signal transduction pathway. Abbreviations are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3K, phosphotidylinositol-3-kinase; PDK, phosphotidylinositol-3, 4, 5-triphosphate-dependent kinase; EGCG, epigallocatechin-3-gallate; TF-3, theaflavin-3,3′-digallate. Tea polyphenols EGCG and TF-3 inhibit the growth factor EGF binding to EGFR, block the activation of the PI3K/Akt signal pathway, then reduce the DNA binding capacity of nuclear transcription factor Sp-1 and finally lead to downregulation of FAS.

and TF-3 also block the induction of the inducible nitric oxide synthase by downregulating lipopolysaccharide-induced activity of the transcription factor NFκB in macrophage.[31] Furthermore, EGCG and TF-3 block cell cycle at the G1 phase in MCF-7 human breast carcinoma cells by inhibiting the activities of cyclin-dependent kinases 2 and 4 as well as inducing the expression of the Cdk inhibitors p21 and p27 proteins.[30] Numerous lines of evidence support the fact that tumor promotion can be enhanced by ROS and oxidative mitotic signal transduction and this enhancing effect can be attenuated or suppressed by tea polyphenols. Based on these findings, we have proposed that the cancer chemoprevention by tea polyphenols may occur by modulation of signal transduction pathways.[16,32]

We have demonstrated that tea polyphenols have profound inhibitory effects on FAS through downregulation of the PI3K/Akt/AP-1 signal pathways. Several FAS inhibitors have been shown to be effective antitumor agents.[4,5] FAS is highly expressed in carcinoma, adenoma and in regenerative epithelium and intestinal metaplasia of the stomach.[33] In addition, FAS is also expressed at a markedly elevated level in subsets of the human breast, ovarian, endometrial and prostate carcinomas.[34] Several anabolic enzymes such as DNA polymerase, ribonucleotide reductase, FAS, etc are overexpressed in cell proliferation and tumor development.[35–37] Numerous studies have demonstrated the striking similarity between the tumor promotion process in

cancer induction and lipogenesis in proliferative tissues. Multiple enzyme systems and signaling pathways are involved in these two complicated cellular processes;[38] some of these enzyme systems and signaling pathways are overlapping, while others are functionally different.[39] It appears that FAS is an important biomarker for developed and developing tumors: FAS is also a key enzyme for lipogenesis and developing body weight. It is expected that once we control the activity or transcription of this enzyme, we may control the tumor growth or body weight gain. It is highly possible that FAS could be considered as another important target enzyme for cancer chemoprevention by tea and tea polyphenols.

FAS is responsible for the anabolic conversion of dietary carbohydrate to fatty acids. It functions normally in the liver to make lipids for export to metabolically active tissues. It is striking that normal endometrial glands express FAS highly during the proliferative phase of the menstrual cycle and FAS expression is closely linked to the expression of Ki67, the marker of proliferation and estrogen and progesterone receptors.[34] The close linkage of FAS expression to markers of proliferation suggests that fatty acid synthesis is related to proliferation in the cells. Other studies showing that FAS inhibitors trigger apoptosis in carcinoma cells indicate the functional importance of fatty acid sysnthesis for certain tumors.[40] The bulk of endogenously synthesized fatty acids are incorporated into membrane lipids by proliferating tumor cells.[15,34] FAS may provide essential lipid components of both structural and functional machinery of cell signaling for cell proliferation.

In previous studies, we have demonstrated that cancer cell proliferation is profoundly inhibited by tea polyphenols including EGCG and theaflavins by suppresson of the function of EGF receptor[20,30,32] that lead to the blockade of MAPK- MEK-ERK-ELK signal pathway[41,42] and PI3K-dependent pathway.[43] In the present study, we have shown that FAS expression is significantly inhibited by tea polyphenols also through suppression of the function of the EGF receptor that leads to the inhibition of the PI3K-Akt-Sp1 signal pathway.

METHODS

Chemicals and Reagents

Epigallocatechin-3-gallate (EGCG) was isolated from Longjing green tea.[28] Theaflavin (TF-1), TF-2, a mixture of theaflavin-3-gallate (TF-2a) and theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-gallate (TF-3) were isolated from black tea leaves as described previously.[28] Catechin, epicatechin, epigallocatechin, caffeine, epidermal growth factor, poly(dI–dC) and Ly294002 were purchased from Sigma Chemical Company (St Louis, MO, USA). Antibodies for fatty acid synthase, Akt kinase, ERK1/2 and β-actin were purchased from BD Biosciences (Los Angeles, CA, USA). Antibodies for phospho-Akt (Ser473) and phospho-ERK (Thr202/Tyr204) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody for Sp-1 and double-stranded Sp-1 oligonucleotide were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RT-PCR reagents and polynucleotide kinase were purchased from Promega (Madison, WI, USA). Isotopes were obtained from Amersham (Arlington Heights, IL, USA).

Preparation of Different Tea Extracts

A measure of 100 g of oolong, black and green tea was suspended in 1000 ml of distilled water at 100°C for 30 min. The supernatant were filtered to eliminate chlorophylls and undissolved particles. The filtrates were extracted with 300 ml chloroform three times to eliminate caffeine and pigments. The remaining aqueous phase was then extracted with 500 ml ethyl acetate two times to extract tea polyphenols. The ethyl acetate phase was washed with 2.5% sodium bicarbonate solution at 500 ml followed by distilled water at 500 ml. The crude extracts of tea polyphenol were obtained after evaporating ethyl acetate to dryness in a vacuum rotary evaporator.

Cell Culture and Drug Treatment

Human breast cancer cell line, MCF-7 and HBL-100, were gifts from Professor Ming-Ching Kao (National Defense Medical University, Taipei, Taiwan) and human hepatoblastoma cell line, HepG2 (HB 8065; American Type Culture Collection, Rockville, MD, USA), was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO Life Technologies, Grand Island, NY, USA) at 37°C humidified 5% CO_2 atmosphere. To study the effect of different tea extracts on the expression of fatty acid synthase in MCF-7 cells, the cells were plated in 6-cm dishes at a density of 1×10^6/ml for 18–24 h, then the medium was changed to a fresh one supplemented with 5% FCS. Various doses of tea extracts were added. After 24 h, cells were harvested. To study the effect of EGCG and TF-3 on EGF-induced expression of FAS, cells were under serum starvation for 24 h and various doses of EGCG and TF-3 were administrated for 30 min before EGF (100 ng/ml) or insulin (100 nM; only in the case of Hep G2 cells) was added. After 24 h, cells were harvested. To study the effect of the phosphotidylinositol- 3-kinase inhibitor Ly294002 on EGF-induced expression of FAS, the cultured condition was the same as described above, except that the incubation time of the inhibitor was 2 h.

Expression of Dominant-Negative Akt by Transient Transfection

Vectors (pcDNA3) expressing the kinase-dead forms of Akt (K179A, dnAkt) were kindly provided by Dr Kuo, ML.[44] MCF-7 cells were transiently transfected with dnAkt or pcDNA3 using Lipofectin Reagent (GIBCO, Invitrogen; Grand Island, NY, USA) according to the manufacturer's instructions. After transient transfection, cells were serum-starved for 18 h and then stimulated with 100 ng/ml EGF for 24 h. The expression of FAS was analyzed as described above.

Western Blot Analysis

This was performed as described in a previous report[33] with some modifications. Total protein extracts were prepared in

Gold lysis buffer (10% (v/v) glycerol; 1% (v/v) Triton 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). Each lane was loaded with 50 μg proteins that were separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (6% for FAS, 10% for *β*-actin, Akt, ERK, phospho-Akt, and phospho-ERK) and transferred on to the PVDF membrane (ImmobilonP, Millipore, Bedford, MA, USA). The membrane was blocked with 1% bovine serum albumin dissolved in phosphate-buffered saline (PBS) and probed with different primary antibodies followed by secondary anti-rabbit/goat/mouse IgG conjugated with horseradish peroxidase. The immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL, Amersham).

RT-PCR

Cells were harvested at 12 h after treatment of tea polyphenols and total RNA was isolated by the ISOGEN reagent (Nippon Gene Company, Tonya-machi, Toyama, Japan). A weight of 5 μg total RNA was reverse transcripted into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) 18 primer by incubating the reaction mixture (25 μl) at 40°C for 90 min. The polymerase chain reaction (PCR) was performed in a final volume of 50 μl containing dNTPs (each at 200 μM), 1 × reaction buffer, 1 μM each primer (FAS: forward: 5′-CTGCAACACCTTCTGCAGTTCTG-3′, reverse: 5′-TCGAATTTGCCAATTTCCAGGAAGC-3′). 2 μl of reverse transcriptase (RT) product and 50 U/ml *Taq* DNA polymerase. After an initial denaturation for 5 min at 95°C, 30 cycles of amplification (95°C for 30 s, 58°C for 2 min and 72°C for 2 min) were performed followed by a 7-min extension at 72°C. A volume of 5 μl of each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining (250-bp FAS fragment).

Electrophoretic Mobility Shift Assay of Sp-1

Cells were harvested and nuclear fractions were prepared as described.[33] Cells were suspended in a hypotonic buffer containing 10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.05% NP-40 (v/v), 1 μg/ml aprotinin and 1 μg/ml leupeptin. The nuclei were pelleted by centrifugation at 1100 rpm for 10 min, 4°C. Nuclei lysis was performed with a hypertonic buffer containing 30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin. After incubation on ice for 30 min, the supernatants containing nuclear protein were obtained by centrifugation at 12 000 *g* for 20 min, 4°C. The nuclear proteins were also retained at −70°C for use in the DNA binding assay. A measure of 5 μg of nuclear protein extract was mixed with salmon DNA in a binding buffer containing 75 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 10% glycerol, then double-stranded Sp-1 oligonucleotide, 5′-ATTCGATCGGGGCGGGGCGAGC-3′ end-labeled by [γ-^{32}P]-dATP (the sequence -CGGGGCGGGGC- is the binding site for Sp-1) was added into the mixture incubated on ice for 30 min. The DNA–protein complex was separated from the free probe on a 5% nondenaturing TBE polyacrylamide gel in 0.5 × Tris/borate/EDTA buffer (TBE: 44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA). Electrophoresis was performed under 120 V for 3 h. The gel was dried and subjected to autoradiography.

Radioisotope labeling studies for lipid biosynthesis

The labeling studies were performed as described by Pizer *et al*.[45] with some modifications. Cells were incubated with 1 μCi (U-^{14}C acetyl-CoA) for 2 h and scraped off plates into an eppendorf tube. Cell lipids were extracted by chloroform and methanol (v/v = 2:1); ions, proteins and hydrophilic substances were removed by distilled water. The organic phase was evaporated by flushing with N_2 stream and redissolved in 20 μl chloroform and then spotted on TLC plastic sheets silica gel 60 F254 (MERCK, Darmstadt, Germany). The mobile phase was hexane : ethyl ether : acetic acid = 90 : 10 : 1. The TLC plastic sheet was dried and subjected to autoradiography. The labeled lipids including cholesterol ester, triglyceride, free fatty acid, free cholesterol and phospholipids on the plastic sheets from top to the bottom were identified as described.[46]

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DUALITY OF INTEREST

None declared.

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