The anaphthoquinone inhibits fatty acid synthase expression in EGF-stimulated human breast cancer cells via the regulation of EGFR/ErbB-2 signaling

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Received 8 May 2006; revised 28 September 2006; accepted 24 October 2006
Available online 10 November 2006

Abstract

Fatty acid synthase (FAS) is a major lipogenic enzyme catalyzing the synthesis of long-chain saturated fatty acids. Most breast cancers require lipogenesis for growth. Here, we demonstrated the effects of anaphthoquinone (TNQ), a member of the thearubigins generated by the oxidation of theaflavin (TF-1), on the expression of FAS in human breast cancer cells. TNQ was found to suppress the EGF-induced expression of FAS mRNA and FAS protein in MDA-MB-231 cells. Expression of FAS has previously been shown to be regulated by the SREBP family of transcription factors. In this study, we demonstrated that the EGF-induced nuclear translocation of SREBP-1 was blocked by TNQ. Moreover, TNQ also modulated EGF-induced ERK1/2 and Akt phosphorylation. Treatment of MDA-MB-231 cells with PI 3-kinase inhibitors, LY294002 and Wortmannin, inhibited the EGF-induced expression of FAS and nuclear translocation of SREBP-1. Treatment with TNQ inhibited EGF-induced EGFR/ErbB-2 phosphorylation and dimerization. Furthermore, treatment with kinase inhibitors of EGFR and ErbB-2 suggested that EGFR/ErbB-2 activation was involved in EGF-induced FAS expression. In constitutive FAS expression, TNQ inhibited FAS expression and Akt autophosphorylation in BT-474 cells. The PI 3-kinase inhibitors and tyrosine kinase inhibitors of EGFR and ErbB-2 also reduced constitutive FAS expression. In addition, pharmacological blockade of FAS by TNQ decreased cell viability and induced cell death in BT-474 cells. In summary, our findings suggest that TNQ modulates FAS expression by the regulation of EGFR/ErbB-2 pathways and induces cell death in breast cancer cells.

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Keywords: Fatty acid synthase; Theanaphthoquinone; SREBP-1; EGFR; ErbB-2

Introduction

Breast cancer is a major cancer of women in the United States and Western Europe (Jemal et al., 2005). Early diagnosis and continuing new therapeutic approaches have managed to prevent the epidemic from causing a concomitant increase in death. Nevertheless, the death of patients due to invasive breast cancer remains a sobering fact and indicates the need to understand this disease in greater depth and to develop new interventions, both preventive and therapeutic (Medina, 2005). Overexpression of ErbB-2 is found in approximately 30% of human breast cancers and correlates with more aggressive tumors and greater resistance to hormone therapy than ErbB-2-negative tumors (Menard et al., 2000). Activation of fatty acid synthase (FAS) expression through modulation of SREBP-1 has been reported in human breast cancer (Yang et al., 2003).

The biosynthetic enzyme fatty acid synthase (FAS) is the major enzyme required for the anabolic conversion of dietary carbohydrates to fatty acids, and it functions normally in cells with high lipid metabolism. In de novo lipogenesis, FAS catalyzes all reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate (Sul and Wang, 1998; Wakil, 1989). FAS provides proliferating cells for endogenously synthesized fatty acid for membrane phospholipid or for other functions (Pizer et al., 1996b; Jackowski et al., 2000). Therefore, a substantial subset of common human cancers— including cancers of the prostate, breast, ovary, colon, thyroid and...
Fig. 1. Chemical structures of theaflavins and theanaphthoquinone (TNQ).
endometrium – have been found to express high levels of FAS (Gansler et al., 1997; Shurbaji et al., 1992; Pizer et al., 1998; Swinnen et al., 1997a). Several recent studies have demonstrated transcriptional regulation of the gene for the enzymes of fatty acid synthesis, including FAS, by sterol regulatory element-binding proteins (SREBPs), sometimes in the context of cell growth (Heemers et al., 2001; Magana and Osborne, 1996; Magana et al., 2000; Swinnen et al., 1997b, 2000a). The FAS promoter contains two independent SREBP binding sites that flank an E-box between bases −73 and −54 that confers SREBP responsiveness (Magana and Osborne, 1996). Remarkably, those studies have shown that FAS inhibition leads to cytostatic and cytotoxic effects in cultured tumor cells and significant antitumor effects in human breast cancer xenografts (Brusselmans et al., 2005; Kuhajda et al., 1994; Pizer et al., 1996a, 2001). Thus, pharmacological inhibition of tumor-associated FAS hyperactivity is under investigation as a potential chemotherapeutic target in established carcinomas.

Tea (Camellia sinensis) is the world’s most popular beverage. Black tea, obtained by fermentation of tea leaves, contains polyphenols including theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3) (Fig. 1). It has been reported that black tea polyphenols have inhibited extracellular signals (Lin et al., 1999; Liang et al., 1999), inhibited cell growth and induced apoptosis (Yang et al., 2000). Black tea extract has been shown to be potent in inhibiting tumorigenesis in animal model systems, including skin (Javed et al., 1998), lung (Yang et al., 1997), colon (Weisburger et al., 1998), and mammary gland (Weisburger et al., 1997; Rogers et al., 1998). Furthermore, polyphenol oxidase in tea oxidizes catechins into quinones and then condenses to form thearubigins in the tea fermentation process (Yang and Koo, 1997). Thearubigins are the most abundantly occurring of the polyphenolic oxidation products in black tea, but their structures and biological activities are not well understood. Theanaphthoquinone (TNQ, Fig. 1) is a member of the thearubigins and is generated by the oxidation of TF-1 during black tea fermentation (Tanaka et al., 2001, 2002). Our recent studies have indicated that TF-1 may inhibit IKK1 activity in macrophages (Pan et al., 2000) and suppress EGF binding to EGFR in A431 cells (Liang et al., 1999). Here, we further study the biological activities of TNQ, a TF-1 derivative, in breast cancer cells.

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) includes EGFR (ErbB, HER1), ErbB-2 (Neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4). All share a similar structural topology and close amino acid sequence homology (van der Geer et al., 1994). The EGFR family of RTKs couples binding of extracellular growth factor ligands to intracellular signaling pathways regulating diverse biological responses, including proliferation, differentiation, cell motility, and survival (Citri and Yarden, 2006). Our previous study indicated that black tea polyphenols possess an antiproliferative activity on tumor cells. These compounds may block the EGF from binding to the EGF receptor and thus suppress mitogenic signal transduction (Liang et al., 1999). Black tea polyphenols also strongly suppress aromatase activity, and they could act as an ErbB-2 tyrosine kinase inhibitor, and attenuate tamoxifen-resistant breast tumors in estrogen-independent breast cancer cells (Way et al., 2004). Ligand binding to RTKs induces receptor homo- and heterodimerization, followed by receptor transphosphorylation and activation (Gamett et al., 1997; Lemmon et al., 1997; Tzahar et al., 1996). ErbB-2 is a preferential candidate to form heterodimeric complexes with ligand-activated EGFR, ErbB-3, and ErbB-4 and plays a critical role in mitogenesis (Sweeney et al., 2001; Crovello et al., 1998; Tzahar et al., 1997). Aberrant activation or overexpression of the EGFR family may play a role in the carcinogenesis of various human tumors and is correlated with poor patient prognosis.

In this study, we have investigated the molecular mechanism of TNQ on EGF-induced expression of FAS in human breast cancer cells. Our data showed that TNQ inhibited EGF-induced expression of FAS and nuclear translocation of SREBP-1 in MDA-MB-231 cells. These effects were down-regulated by the PI 3-kinase/Akt signal pathway. Moreover, our data also showed that EGFR/ErbB-2 signaling was involved in TNQ-inhibited EGF-induced expression of FAS. TNQ inhibited constitutive FAS expression and induced cell death in the overexpression of ErbB-2 breast cancer cells. Taken together, these findings indicate that TNQ-inhibiting EGF-induced expression of FAS is associated with the suppression of EGFR/ErbB-2 signaling.

Materials and methods

Materials. Theanaphthoquinone (TNQ) was synthesized from (−)-epicatechin (EC) and (−)-epigallocatechin (EGC) (Tanaka et al., 2002; Joo et al., 2005). EGF, PD98059, LY294002, and Wortmannin were purchased from Sigma Chemical (St Louis, MO). PD153035 and AG825 were purchased from Calbiochem (La Jolla, CA). Anti-FAS antibody was from BD Biosciences (Los Angeles, USA). Anti-phospho-(Ser473)-Akt and anti-Akt were from Cell Signal Technology (Beverly, MD), and anti-phosphotyrosine (4G10) was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-ERK, anti-ERK, anti-EGRF, anti-SREBP-1, and anti-histone were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-ErbB-2 was from Oncogene Science (Manhasset, NY).

Cell culture and treatment. MDA-MB-231 and BT-474 human breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in DMEM/F12 (Invitrogen, Grand Island, NY), and BT-474 cells were maintained in RPMI 1640 (Sigma Chemical, St Louis, MO). All of the cells were supplemented with 10% fetal calf serum (FCS, HyClone Laboratories, Logan, UT) and 100 U penicillin–streptomycin. Cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After reaching confluence, cells were seeded and then incubated with serum-free medium for 24 h. Then cells were pretreated with TNQ, PI 3-kinase, MAP kinase, EGFR or ErbB-2 kinase inhibitor for 30 min and then stimulated with EGF (50 ng/ml). Cells were harvested, and Western blot analyses, RT–PCR and immunoprecipitation were performed.

Western blot analysis. Cells were plated in normal growth medium in a 100-mm cell culture dish and then incubated with serum-free medium for 24 h. Following treatment, cells were rinsed twice in ice-cold PBS and scraped into an appropriate extraction buffer (10 mM Tris–HCl, pH 7; 140 mM sodium chloride; 3 mM magnesium chloride; 0.5% [v/v] NP-40; 2 mM phenylmethylsulfonyl fluoride; 1% [w/v] aprotinin; and 5 mM dithiothreitol) for 30 min on ice. The extracts were centrifuged for 30 min at 12000 g. Proteins were loaded at 50 μg/lane on sodium dodecyl sulfate (SDS)–polyacrylamide gel, and then
transferred to nitrocellulose membranes. The membranes were blocked for 30 min at room temperature in PBS plus 0.5% Tween 20 containing 1% bovine serum albumin, then incubated for 2 h at room temperature with a 1:1000 dilution of one of the following mouse monoclonal antibodies against human, i.e., FAS, Akt, ERK, and β-actin, and rabbit polyclonal antibodies against human, i.e., phospho-Akt, phospho-ERK, SREBP-1, and histone. After washing, the membranes were incubated for 60 min at 25 °C with the 1:3000 dilution of an appropriate horseradish peroxidase-labeled secondary antibody and a bound antibody visualized by chemiluminescence detection (Amer sham, Aylesbury, UK). Actin or histone was used as the internal control. Quantitative data were obtained using a computing densitometer with Image-Pro plus software. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of actin or histone. The density of the band was then expressed as a relative density compared to that in untreated cells (control), which was taken as one-fold.

Immunoprecipitation. Cell lysates were prepared and clarified as above. Lysates containing 500 μg protein were then incubated with the appropriate amount of EGFR or ErbB-2 antibody at 4 °C for 3 h. About 50 μl of a 1:1 slurry of protein A/G–agarose were then added and the samples were rotated overnight. Immunoprecipitations were washed twice in lysis buffer, followed by a final wash in Tris-buffered saline. Captured proteins were then eluted by boiling the beads in 100 μl SDS sample buffer for 5 min. Samples were then run on 8% SDS–PAGE, transferred to nitrocellulose and blotted with anti-phosphoryrosine antibodies (4G10, 1:1000 dilution). In each case, blots were stripped and reprobed with the antibody used for immunoprecipitation.

Preparation of nuclear extracts. Cells were plated in a normal growth medium in a 100-mm cell culture dish before being incubated with serum-free medium for 24 h. Following treatment, cells were rinsed twice in ice-cold PBS and scraped into 200 μl hypotonic extraction buffer (10 mM sodium phosphate buffer pH 7.0, 10 mM sodium fluoride, 5 mM EDTA, 1 mM PMSF, 1% NP-40, and 5 mM magnesium chloride) for 15 min on ice. The extracts were centrifuged for 30 min at 12,000g. The nuclei were washed twice with 400 μl hypotonic buffer, and an appropriate hypotonic extraction buffer (10 mM sodium phosphate buffer pH 7.0, 10 mM sodium fluoride, 5 mM EDTA, 1 mM PMSF, 1% NP-40, 5 mM magnesium chloride, and 0.1% sodium chloride) was added for 30 min on ice. The extracts were centrifuged for 30 min at 12,000g. The supernatant containing nuclear protein was collected and stored at −70 °C after the determination of protein concentrations.

RT–PCR. Following the drug treatment, the cells were washed in ice-cold PBS, and total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction according to the manufacturer’s instructions (Nippon Gene, Japan). The cDNA was prepared from the total RNA (5.0 μg) with reverse transcriptase and oligo(dT)18 primer at 42 °C for 60 min. The PCR was performed in a final volume of 25 μl containing dNTPs (each at 200 μM), 1.0× reaction buffer, 2.0 μg cDNA product, and 50 U/ml proκ PO (Promega, Madison, WI). PCR primers for FAS and GAPDH were synthesized according to the following oligonucleotide sequences: FAS, forward primer 5′-GAAACTGCAGGAGCTGCT-3′, reverse primer 5′-CAGGAGGTCGAGGCTACAGC-3′; GAPDH, forward primer 5′-GTAAGGTCCGTTGAGACGGATTGTCG-3′, reverse primer 5′-CATGATGCCATGAGGCTACAC-3′. After an initial denaturation for 10 min at 95 °C for FAS PCR, 40 cycles of amplification (95 °C for 15 s, 56 °C for 60 s, and 72 °C for 60 s) were performed, followed by a 10-min extension at 72 °C. The PCR products were separated by electrophoresis on 1.0% agarose gel and visualized by ethidium bromide staining (Yang et al., 2003).

Determination of cell viability. Human breast cancer BT-474 cell lines were plated at a density of 1 × 10⁶ cells in 48-well plates and allowed an overnight period for attachment. The medium was then removed, and fresh medium, along with various concentrations of TNQ, was added to cultures in parallel. After treatment, the cell viability was determined by the conventional MTT reduction assay. This assay relies primarily on the mitochondrial metabolic capacity of viable cells and hence reflects the intracellular redox state. After incubation, cells were treated with the MTT solution (final concentration, 0.25 mg/ml) for 2 h at 37 °C. The dark purple formazan crystals formed in the intact cells were dissolved with DMSO, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

Flow cytometry. BT-474 cells (2 × 10⁶) were cultured in 60-mm cell culture dishes for 24 h. Then cells were transferred to fresh medium and treated with various concentration of TNQ for 24 h. After 24 h of treatment, cells were harvested, washed with PBS, resuspended in 200 μl of PBS, and fixed in 800 μl of icid 95% ethanol at −20 °C. After being left to stand overnight, cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at 37 °C for 30 min. Then 1 ml of propidium iodide solution (50 μg/ml) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescence dye using a FACScan Flow Cytometer (BD Biosciences, San Jose, CA).

Statistical analysis. The results were expressed as mean ± SE calculated from the specified numbers of determination. A Student’s t-test was used to compare individual data with control value. A probability of p < 0.05 was taken as denoting a significant difference from control data.

Results

Effect of TNQ on EGF-induced FAS expression in human breast cancer MDA-MB-231 cells

To investigate the effect of theanaphthoquinone (TNQ) on EGF-induced FAS expression in human breast cancer cells, we treated MDA-MB-231 breast cancer cells with TNQ in the presence of EGF. MDA-MB-231 cells were cultured in serum-free medium for 24 h, and then treated with EGF (50 ng/ml) for 24 h. Cell extracts were prepared and analyzed for FAS protein expression by Western blot analysis. As demonstrated in Fig. 2A, treatment with EGF (50 ng/ml) led to an increase of FAS protein level in MDA-MB-231 breast cancer cells. Pretreatment with TNQ (10, 20, and 40 μM) for 30 min followed by EGF stimulation for 24 h resulted in a dose-dependent inhibition of EGF-induced FAS protein level. Theaflavin (TF-1), the reductive form of TNQ, also exhibited the inhibition of EGF-induced FAS protein expression in MDA-MB-231 while TNQ showed more potent inhibition (Fig. 2A).

To further examine whether augmentation or inhibition of EGF signaling would modulate the transcriptional level of FAS, MDA-MB-231 cells were cultured in serum-free medium and pretreated with 20 μM TNQ or TF-1 for 30 min followed by the presence of EGF for 12 h. Because the maximum mRNA level of FAS was achieved after 12 h of EGF stimulation (data not shown), we created these conditions to examine the effects of TNQ or TF-1 on EGF-induced mRNA levels of FAS. Afterward, total RNA was prepared for RT–PCR. As shown in Fig. 2B, exposure to EGF for 12 h induced the expression of FAS mRNA. The TNQ-treatment decreased EGF-induced expression of FAS mRNA, but TF-1 showed slight enhancement. These results suggested that EGF increased the expression of FAS mRNA while TNQ treatment inhibited it. On the other hand, TF-1 might suppress FAS expression by inhibiting its translation rather than its transcription. Further investigation is needed to clear this point.
Effect of TNQ on EGF-induced SREBP-1 translocation in human breast cancer MDA-MB-231 cells

Important mediators of FAS transcriptional regulation in liver and adipose tissue include SREBPs, which bind to sterol regulatory elements (SRE) located in the promoters of target genes. Activation by the SREBP pathway by typical inducers such as cholesterol depletion, involves proteolytic cleavage of SREBP precursors, ultimately resulting in an increase in nuclear SREBP (Rawson, 2003; Horton et al., 2002). To examine whether EGF treatment also increases the nuclear content of SREBP-1 in our experiment, MDA-MB-231 cells were treated with EGF for different periods of time, and nuclear extracts were prepared. These extracts were subjected to Western blot analysis with anti-SREBP-1 antibody. As demonstrated in Fig. 3A, the nuclear content of SREBP-1, apparent as a 66 kDa protein, was increased 60 min after EGF treatment and remained elevated for at least 3 h.

To further investigate whether TNQ inhibited EGF-induced translocation of SREBP-1, MDA-MB-231 cells were cultured in serum-free medium for 24 h and then pretreated with various concentrations of TNQ for 30 min before EGF treatment for 1 h. Nuclear extracts were prepared, and Western blot analysis with anti-SREBP-1 was performed. As shown in Fig. 3B, TNQ inhibited EGF-induced translocation of SREBP-1.

Effects of TNQ on EGF-induced ERK1/2 and Akt activation in human breast MDA-MB-231 cells

Growth factor, such as EGF, is known to up-regulate a number of signal transduction pathways, including the MEK-1/ERK1/2 MAP kinase and PI 3-kinase/Akt pathways. To determine the effect of TNQ on EGF-activated ERK1/2 and Akt signals, Western blot analysis with antibodies specific to phospho-ERK1/2 and phospho-Akt was performed. As shown in Fig. 4, EGF induced a time-dependent increase of ERK1/2 MAP kinase in MDA-MB-231 cells. Pretreatment with TNQ for 30 min inhibited the phosphorylation of ERK1/2 in EGF-treated cells for 5, 15, 30 min (Fig. 4A).

We next examined the effect of TNQ on the Akt signaling pathway, a pathway critical for cell survival. Activation of Akt, assessed by phospho-Ser473 immunoreactivity, was detected in serum-starved MDA-MB-231 cells. Our results indicated that treatment with EGF led to a time-dependent activation of Akt, and this response was blocked when cells were pretreated with TNQ (Fig. 4B).

Down-regulation of PI 3-kinase-Akt signaling pathway reduces EGF-induced FAS expression and SREBP-1 translocation in human breast MDA-MB-231 cells

Nutritional and hormonal regulation of FAS expression in lipogenic cells occur through a modulation of the SREBP-1, which is known to be regulated by MEK-1/ERK1/2 MAP kinase and PI 3-kinase/Akt signaling cascades (Fleischmann and Iynedjian, 2000; Kotzka et al., 2000). To evaluate the relative importance of MEK-1/ERK1/2 MAP kinase and the PI 3-kinase/Akt signaling pathway to the EGF-induced translocation of SREBP-1, MDA-MB-231 cells were treated with several inhibitors of PI 3-kinase and ERK1/2—such as LY294002, Wortmannin, or PD98059 for 30 min before EGF stimulation for 1 h. Nuclear extracts were prepared for Western blot analysis.
with anti-SREBP-1 antibody. The data in Fig. 5A indicate that treatment with 40 μM TNQ, 20 μM LY294002, and 1 μM Wortmannin blocked EGF-induced nuclear translocation of SREBP-1. However, ERK1/2 inhibitor, PD98059, had no effect on it. To further confirm whether blockade of the EGF-induced PI 3-kinase/Akt/SREBP-1 pathway may inhibit FAS expression, MDA-MB-231 cells were pretreated with the PI 3-kinase and ERK1/2 inhibitors (20 μM LY294002, 1 μM Wortmannin, or 50 μM PD98059) for 30 min before EGF was induced for 24 h. Whole cell extracts were prepared for Western blot analysis with anti-FAS antibody. As shown in Fig. 5B, treatment with PI 3-kinase inhibitors, LY294002 and Wortmannin, blocked the EGF-induced expression of FAS, but ERK1/2 inhibitor, PD98059, had no effect. This data suggested that EGF-induced FAS expression was indeed regulated by the PI 3-kinase/Akt/SREBP-1 pathway, and the blockade of FAS expression by TNQ also down-regulated the PI 3-kinase/Akt signaling pathway.

TNQ inhibition of EGF-induced FAS expression was through suppressing tyrosine phosphorylation and dimerization of EGFR/ErbB-2

The EGF family of ligands – which includes EGF, transforming growth factor α, and heregulin – stimulated the formation of receptor homodimers and heterodimers and

![Fig. 3. Inhibitory effects of TNQ on EGF-induced SREBP-1 translocation in MDA-MB-231 breast cancer cells. (A) Cells were treated with 50 ng/ml of EGF for 10, 20, 30, 60, 120, and 180 min or (B) pre-treated with indicated concentrations of TNQ for 30 min before incubation with 50 ng/ml of EGF for 1 h. Nuclear extracts were prepared for Western blot analysis with anti-SREBP-1 and anti-histone antibody as described in Materials and methods. The extent of SREBP-1 and histone protein expression was quantified using a densitometer with Image-Pro Plus software. The relative level was calculated as the ratio of SREBP-1 to histone protein levels. Results are expressed as the mean±SE (n=3). *p<0.05 compared with at the time 0 group or EGF-treated group.](image1)

![Fig. 4. Inhibitory effects of TNQ on EGF-induced phosphorylation of Akt and ERK in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were incubated in serum-free medium for 24 h before being stimulated with 50 ng/ml of EGF for the indicated times. For each time point, cells received prior treatment with DMSO (0.01%) or TNQ (40 μM) for 30 min. Whole cell lysates were then prepared and equivalent protein amounts (40 μg) subjected to Western blot analysis using antibodies directed against (A) phospho-ERK or (B) phospho-Akt as described in Materials and methods. The blot was then stripped and reprobed with total ERK or total Akt antibodies. The extent of p-ERK or p-Akt and total ERK or total Akt protein expression was quantified using a densitometer with Image-Pro Plus software. The relative level was calculated as the ratio of p-ERK or p-Akt to total ERK or total Akt protein levels, respectively. Results are expressed as the mean±SE (n=3). *p<0.05 compared with the EGF-treated group.](image2)
tyrosine kinase activity (Citri and Yarden, 2006). EGF preferentially binds to EGFR, inducing predominantly EGFR homodimers or EGFR/ErbB-2 heterodimers. After receptor tyrosine autophosphorylation occurs several intracellular substrates are involving in the Ras/Raf/MEK-1/ERK1/2 MAP kinase, the PI 3-kinase/Akt, and other signaling pathways that regulate multiple biological processes, including apoptosis and cellular proliferation (Heldin, 1995; Hubbard et al., 1998). There is evidence of molecular cross-talk between the ErbB-2 and FAS signaling pathway at the level of transcription, translation, and biosynthetic activity (Kumar-Sinha et al., 2003). To investigate whether TNQ retains effects on EGFR/ErbB-2-regulated FAS expression, we chose BT-474 breast cancer cell lines, which lower expression of EGFR and raise expression of ErbB-2 compared with MDA-MB-231 cells (Anderson et al., 2001; Menendez et al., 2004a). Cells were grown in serum-free medium for 24 h and then incubated with 40 μM of TNQ for 30 min before being stimulated with 50 ng/ml of EGF for 5 min. After incubation, immunoprecipitation with antibodies specific for ErbB-2 or EGFR was performed. The immunoprecipitates were then examined by immunoblot analysis with an anti-phosphotyrosine antibody. As shown in Figs. 6A and B, EGF induced tyrosine phosphorylation of ErbB-2 and EGFR, and pre-incubation with TNQ inhibited this phosphorylation. To determine whether TNQ inhibited EGF-induced ErbB-2 activation due to EGFR/ErbB-2 heterodimerization disruption, we reprobed with anti-EGFR antibodies. As shown in the middle panel of Fig. 6A, EGFR was associated with ErbB-2 after stimulation with EGF for 5 min, but pretreatment with TNQ for 30 min inhibited EGF-induced EGFR/ErbB-2 dimerization. These data suggest that EGF induces the heterodimerization of EGFR/ErbB-2, followed by EGFR and ErbB-2 transphosphorylation on tyrosine residues. TNQ inhibited EGF-induced EGFR and ErbB-2 activation in order to disrupt the heterodimerization of EGFR/ErbB-2.

To further examine the relationship between EGF/ErbB-2 and FAS expression, MDA-MB-231 and BT-474 cells were pretreated with 1 μM of the tyrosine kinase inhibitors of EGFR and ErbB-2, namely PD153035 and AG825, respectively, for 30 min before EGF treatment for 24 h. Cell extracts were prepared for Western blot analysis with anti-FAS antibody. As shown in Fig. 6C, inhibition of the tyrosine kinase of EGFR or ErbB-2 by their kinase inhibitor down-regulated EGF-induced FAS expression.

Inhibition of constitutive FAS expression on ErbB-2 and EGFR overexpressing breast cancer cell via TNQ

Based on the data discussed above showing that TNQ inhibits EGF-induced EGFR/ErbB-2/PI 3-kinase/Akt/SREBP-1 activation, we assessed the effect of TNQ alone on this signal cascade in BT-474. First, we assessed the effect of TNQ alone on constitutive FAS expression. As shown in Fig. 7A, TNQ treatment of BT-474 cells for 24 h resulted in the reduction of FAS expression in a dose-dependent manner. Furthermore, we investigated the effect of TNQ on Akt and ERK activation. As shown in Fig. 7B, TNQ treatment of BT-474 cells for 12 h resulted in a decrease of
phospho-Ser\textsuperscript{473}-Akt in a dose-dependent manner, but total Akt protein decreased after the 40 μM treatment. Treatment of cells with 10 ng/ml of EGF (+) or nothing (−) for 5 min. After stimulation, cells were harvested and equal amounts of total protein were then subjected to immunoprecipitation using (A) anti-ErbB-2 or (B) anti-EGFR antibody. The immunoprecipitates were then subjected to gel electrophoresis and Western blotting with anti-phospho-tyrosine as described in Materials and methods. The blot of (A) was then stripped and reprobed with EGFR. (C) MDA-MB-231 and BT-474 cells were incubated in serum-free medium for 24 h and then pre-treated with 1 μM PD153035 or 1 μM AG825 for 30 min before being stimulated with 50 ng/ml EGF for 24 h. FAS expression was measured by Western blot using anti-FAS antibody as described in Materials and methods. The extent of FAS and actin protein expression was quantified using a densitometer with Image-Pro Plus software. The relative level was calculated as the ratio of FAS to actin protein levels. Results are expressed as the mean±SE (n=3). *p<0.05 compared with the EGF-treated group.

To further investigate whether constitutive FAS expression was down-regulated in the EGFR/ErbB-2-dependent mechanism, BT-474 cells were treated with tyrosine kinase inhibitors of EGFR or ErbB-2 for 24 h, and Western blot analysis with anti-FAS antibody was performed. As shown in Fig. 7D, inhibition of tyrosine kinase of EGFR or ErbB-2 by their kinase inhibitor reduced constitutive FAS expression. These results suggest that TNQ down-regulated constitutive FAS expression by inhibition of the EGFR/ErbB-2/PI 3-kinase/Akt pathway.

Effects of TNQ on cell viability and induction of cell death in breast cancer cells

To evaluate whether the cancer cell cytotoxicity induced by TNQ was related to the inhibition of ErbB receptor-mediated FAS signaling, we analyzed the cytotoxic efficacy of TNQ in BT-474 cells expressing high levels of ErbB-2. BT-474 cells were exposed for 24, 48, 72 h to TNQ (10, 20, 40 μM), and cell viabilities were observed by MTT assay. As shown in Fig. 8A,
treatment with TNQ markedly decreased BT-474 cell viability in a dose- and time-dependent manner. To further assess whether TNQ induced cell growth arrest or cell death, BT-474 cells were treated with TNQ (10, 20, and 40 μM) for 24 h. Cells were harvested, and flow cytometry analysis was performed. As shown in Fig. 8B, treatment with TNQ for 24 h was significantly induced cell death in BT-474 cells in a dose-dependent manner. The percentage of dead BT-474 cells was 2.5±1.2%, 3.6±0.3% (p>0.05), 8.2±0.2% (p<0.05), and 21.1±0.3% (p<0.05) after 24 h of treatment with 0, 10, 20, and 40 μM of TNQ (Fig. 8C). These data indicated that TNQ contributed to BT-474 cell death through down-regulation of FAS expression.

Discussion

In this study, we investigated the effects of the arbutin (TNQ), a member of the thearubigins generated by the oxidation of theaflavin (TF-1), on FAS expression. First, we found that EGF-induced the expressions of FAS mRNA and protein were reduced by TNQ. EGF-induced nuclear translocation of SREBP-1 was also blocked by TNQ in a dose-dependent manner. Accordingly, we examined the effects of TNQ on EGF-induced Akt and ERK1/2 activation and found both were inhibited by TNQ. We have further demonstrated that the EGF-induced nuclear translocation of SREBP-1 and expression of FAS were reduced by inhibitors of PI 3-kinase/Akt. TNQ also inhibited EGF-induced EGFR and ErbB-2 activation. Treatments of tyrosine kinase inhibitors of EGFR or ErbB-2 resulted in down-regulation of EGF-induced FAS expression. Our data also indicated that TNQ inhibited constitutive FAS expression and induced cell death in ErbB-2-overexpressed cells.

Thearubigins are the most abundant phenolic fraction of black tea, and their structures and biological activities are not well characterized. TNQ, a member of the thearubigins, was identified as an oxidation product of TF-1 using DPPH and...
peroxidase/hydrogen peroxide oxidant systems (Tanaka et al., 2002; Jhoo et al., 2005). The time course of the oxidation of a mixture of EC and EGC with a banana homogenate showed that the concentration of TF-1 began to decrease when EGC was exhausted. At the same time TNQ appeared in the reaction mixture (Tanaka et al., 2002). These results suggest that TNQ is biosynthesized from TF-1 during tea fermentation. There is no report addressing the issue about the biological activities of TNQ. Therefore, we are interested in comparing the effects of TNQ and TF-1 on the regulation of FAS expression. Our results suggest that TNQ had a more potent inhibitory effect than TF-1 on the EGF-induced expression of FAS protein and mRNA (Fig. 2).

Previously, several studies demonstrated that growth factor and androgens stimulate FAS gene expression through steroid regulatory element binding proteins (SREBPs) in prostate cancer cells lines (Swinnen et al., 1997a, 2000a,b; Heemers et al., 2001). Rossi et al. have demonstrated a strong correlation between the expression of FAS and expression of its transcriptional regulator, SREBP-1, in both primary and metastatic androgen-independent human tumors analyzed by oligonucleotide arrays (Rossi et al., 2003). Accordingly, we further examined the effects of TNQ on EGF-induced nuclear translocation of SREBP-1. We found that EGF induced nuclear translocation of SREBP-1, and treatment with TNQ in EGF-stimulated cells inhibited nuclear translocation of SREBP-1 (Fig. 3B). In cancer cells, SREBP-1 expression will be driven by a constitutive activation of MAPK and PI 3-kinase/Akt signaling pathways in response to oncogenic changes, including overexpression of growth factors (e.g. EGF, heregulin) and/or the overexpression of growth factor receptors (e.g. ErbB-2, EGFR).

In our study, TNQ treatment can reduce EGF-induced Akt and ERK activation in MDA-MB-231 cells (Fig. 4). To further examine roles of PI 3-kinase/Akt and MEK-1/ERK1/2 MAP kinase in FAS regulation, we treated MDA-MB-231 cells with LY294002, Wortmannin, or PD98059 before stimulation with EGF. Our data indicate that inhibition of the PI 3-kinase/Akt pathway down-regulated EGF-induced nuclear translocation of SREBP-1 and FAS expression (Fig. 5). Studies have shown that Sp-1 and NF-Y, together with SREBP-1, are essential to the sterol regulation of the human FAS gene (Xiong et al., 2000). A recent study of ours also showed that EGCG inhibited DNA binding of Sp-1 and subsequently, suppressed FAS expression in MCF-7 cells (Yeh et al., 2003). Thus, the effects of TNQ on the regulation of Sp-1 or NF-Y in our system cannot be excluded. Further investigation the effects of TNQ on Sp-1- and NF-Y-regulated FAS expression should be undertaken.

The genes/proteins regulated by ErbB-2-mediated signal transduction pathways have recently been investigated (Wilson et al., 2002; Mackay et al., 2003; White et al., 2004). Transcriptome analysis of the ErbB-2 (HER-2/neu) oncogene revealed a molecular connection to FAS in human breast cancer cells and the report indicated that ErbB-2 regulated the expression of FAS through the PI 3-kinase/Akt pathway (Kumar-Sinha et al., 2003). Another study also indicated that reducing the ErbB-2 expression by Herceptin significantly decreased FAS protein in SKBr3 cell lines (Zhang et al., 2005). Our data show that EGF-induced EGFR, ErbB-2 phosphorylation, and EGFR/ErbB-2 heterodimerization were all inhibited by TNQ (Fig. 6A and B). To further confirm whether EGFR and ErbB-2 activation were involved in regulating FAS expression, the EGFR and ErbB-2 tyrosine kinase inhibitors, PD153035 and AG825, were used. As shown in Fig. 6C, both treatment with PD153035 and AG825 inhibited EGF-induced FAS expression in MDA-MB-231 and BT-474 cells. Our findings implicate that inhibition of EGF-induced FAS expression by TNQ may be associated with the EGFR/ErbB-2 activation. These results are consistent with recent transcriptome analysis and proteomic study (Kumar-Sinha et al., 2003; Zhang et al., 2005). However, data from studies on the regulation of FAS and ErbB-2 appear inconsistent, with some claiming that ErbB-2 regulates FAS expression via the PI 3-kinase/Akt pathway (Kumar-Sinha et al., 2003; Zhang et al., 2005) and others counter-claiming that FAS inhibited ErbB-2 transcription via up-regulation of PEA3, a transcription repressor of ErbB-2 (Menendez et al., 2004b, 2005).
Our previous study has indicated that tea polyphenols, like EGCG and theaflavins possess an antiproliferative activity on tumor cells. The antiproliferative effect of these compounds can block the EGFR from binding to EGFR receptor and thus suppress mitogenic signal transduction. Blocking of EGFR binding to EGFR receptor maybe due to the similar chemical structure with galloyl group in theaflavins and EGCG (Liang et al., 1999). TNQ does not possess any galloyl groups, but it displayed ability to inhibit EGFR and ErbB-2 activation (Fig. 6). Nakaya and Miyasaka (2003) demonstrated that some quinone derivatives can inhibit RTK activity. Our data implicate that quinone group of TNQ may be involved in the inhibition of EGFR and ErbB-2 activation and then block downstream signaling. How, though, does TNQ block EGFR and/or ErbB-2 activation? We speculate that the inhibition of EGFR activity by TNQ might prevent EGF from binding to its receptor. However, TNQ may interfere with the dimerization of EGFR and/or ErbB-2. It would be pertinent to investigate the mechanism by which TNQ inhibits EGFR and/or ErbB-2 activation.

In the present study, we demonstrate that TNQ inhibited EGFR-induced EGFR/ErbB-2 dimerization, activation, and the PI 3-kinase/Akt/SREBP-1 pathway with a concomitant down-regulation of FAS expression in breast cancer cells. This study provides the basis for a molecular mechanism by which TNQ inhibits EGFR and ErbB-2 activation, leading to suppressed FAS expression in the target cells and induced cell death.

Acknowledgments

We thank Dr. Yu-Chih Liang and Dr. Chiung-Ho Liao for providing excellent technical supports. This work was supported by grants from the National Science Council NSC94-2300-B-002-118, NSC94-2320-B-002-019, NSC95-2320-B-002-111 and NSC95-2321-B-002-016.

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