Original Article

Role of GTP-binding proteins in reversing the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells

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Tocotrienols are a subclass of vitamin E compounds that display potent anticancer activity. Determining the anticancer mechanism of action of tocotrienols will provide essential information necessary for understanding the potential health benefits of these compounds in reducing the risk of breast cancer in women. Epidermal growth factor (EGF) is a potent mitogen for normal and neoplastic mammary epithelial cells. Initial events in EGF-receptor (EGF-R) mitogenic-signalling are G-protein activation, stimulation of adenylyl cyclase and cyclic AMP (cAMP) production. Studies were conducted to determine if the antiproliferative effects of tocotrienols are associated with reduced EGF-induced G-protein and cAMP-dependent mitogenic signalling. Preneoplastic CL-S1 mouse mammary epithelial cells were grown in culture and maintained on serum-free media containing 0–25 µmol/L tocotrienol-rich fraction of palm oil and/or different doses of pharmacological agents that alter intracellular cAMP levels. Tocotrienol-induced effects on EGF-receptor levels of tyrosine kinase activity, as well as EGF-dependent mitogen-activated pathway kinase (MAPK) and Akt activation, were determined by western blot analysis. Results demonstrate that the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells do not reflect a reduction in EGF-receptor mitogenic responsiveness, but rather, result from an inhibition in early post-receptor events involved in cAMP production upstream from EGF-dependent MAPK and phosphoinositide 3-kinase/Akt mitogenic signalling. In summary, these data further characterise the mechanism of action of tocotrienols in suppressing preneoplastic mammary epithelial cell proliferation, and advance the current understanding of the potential health benefits of these compounds in reducing the risk of breast cancer in women.

Key words: Akt, breast cancer, cyclic AMP, G-proteins, phosphoinositide 3-kinase, tocotrienols, vitamin E.

Introduction

Breast cancer is the most prevalent malignancy in women. While specific genes have been identified that predispose women to breast cancer, this inherited form of the disease is rare and accounts for no more than 5–10% of all breast cancers. The vast majority of breast cancers result from factors that are yet to be identified. Although there have been significant advances in breast cancer treatment, therapeutic strategies designed to prevent breast cancer would greatly reduce risk and mortality.

Tocotrienols, a subclass of compounds in the vitamin E family, significantly inhibit mitogen-induced proliferation in preneoplastic and neoplastic mammary epithelial cells. These findings are of particular interest because they were achieved using treatment doses that had no effect on normal mammary epithelial cell growth or viability, suggesting that tocotrienols may have significant value as therapeutic agents for breast cancer prevention and/or treatment. Elucidation of the mechanisms that mediate the antiproliferative effects of tocotrienols would provide essential information necessary for understanding the potential health benefits of these compounds and provide insights for the development of effective therapeutic strategies for the utilisation of tocotrienols in reducing the risk of breast cancer in women.

Although tocotrienols are potent antioxidants, the anticancer activity of these compounds is not dependent on such activity. Recent studies have shown that tocotrienols specifically inhibit epidermal growth factor (EGF)-dependent mitogenesis in preneoplastic and neoplastic mammary epithelial cells. The mitogenic actions of EGF are mediated through specific membrane-bound receptors that contain an extracellular ligand binding domain, a transmembrane hydrophobic segment and a cytoplasmic catalytic domain that contains intrinsic tyrosine kinase activity. EGF activation of the EGF receptor results in receptor autophosphorylation of C-terminal tyrosine residues that are required for interaction and tyrosine phosphorylation of various intracellular substrates associated with mitogenic signal transduction.

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Studies have also shown that the EGF receptor is a G-protein-coupled receptor that activates adenylcyclase, leading to an elevation in intracellular cyclic AMP (cAMP) levels and activation of cAMP-dependent protein kinases in mammary epithelial cells. 8–10 Activation of cAMP-dependent protein kinases is a key event that initiates additional mitogenic cascades, including the phosphoinositide 3-kinase (PI3K/Akt) (also known as PKB) and the mitogen-activated protein kinase (MAPK) pathway. 11 Therefore, it is hypothesised that tocotrienol inhibition of EGF-dependent preneoplastic and neoplastic mammary epithelial cell proliferation results from tocotrienol-induced alterations in cAMP production and/or cAMP-dependent mitogenic signalling.

Experiments were conducted using preneoplastic mouse mammary epithelial cells that were derived from the hyperplastic D1 cell line that spontaneously arose in BALB/c mice. 2,12 The CL-S1 preneoplastic cell line is immortal in culture and forms only hyperplastic nodules, not solid tumours, upon transplantation into the mouse mammary gland fat pad. 2,12 The CL-S1 preneoplastic cell line provides an ideal experimental model for the study of antiproliferative mechanisms of tocotrienols at the initial stages of mammary epithelial cell transformation and tumour progression, and determination of the efficacy of tocotrienols as a chemopreventive agent. The following experiments were conducted to characterise tocotrienol effects on EGF-induced EGF receptor tyrosine kinase activity, cAMP production and activation of cAMP-dependent protein kinases in preneoplastic CL-S1 mammary epithelial cells grown in culture and maintained on serum-free media containing EGF as a mitogen.

Materials and methods
All materials were purchased from Sigma Chemical Company (St Louis, MO, USA), unless otherwise stated. Preneoplastic CL-S1 mammary epithelial cells were serially passaged at subconfluent cell density and maintained in serum-free control media consisting of DMEM/F12 containing 5 mg/mL bovine serum albumin (BSA), 10 µg/mL transferrin, 100 U/mL soybean trypsin inhibitor, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL EGF and 10 µg/mL insulin. For subculturing, cells were rinsed twice with sterile Ca2+- and Mg2+-free phosphate-buffered saline (PBS), and then incubated in 0.05% trypsin containing 0.025% ethylenediaminetetraacetic acid in PBS for 5 min at 37°C. The released cells were then diluted in DMEM/F12 media, pelleted by centrifugation, and the cell pellets then resuspended in serum-free media and counted using a haemocytometer. CL-S1 cells were plated at a density of 1 × 10⁵ cells/well in 24-well culture plates for growth studies, and at a density of 1 × 10⁶ cells/100 mm culture plates for western blot analysis. Cells were divided into different treatment groups fed serum-free control or treatment media containing various doses of 8-Br-cAMP, cholera toxin, pertussis toxin or forskolin alone, or in combination with 0–25 µmol/L of the tocotrienol-rich fraction of palm oil (TRF) every other day and maintained in a humidified incubator at 37°C in an environment of 95% air and 5% CO₂.

The tocotrienol-rich fraction of palm oil was assayed by high-performance liquid chromatography prior to use in experimentation and determined to be composed of 20.2% α-tocopherol, 16.8% α-tocotrienol, 44.9% γ-tocotrienol, 14.8% δ-tocotrienol and 3.2% of a non-vitamin E lipidsoluble contaminant. 2,3 Treatment doses of TRF were then calculated based on the percentage composition and molecular weights of individual vitamin isoforms within TRF. In order to dissolve the highly lipophilic TRF in aqueous culture media, TRF was conjugated to BSA as previously described. 2,3 Briefly, an appropriate amount of TRF was placed in a 1.5-mL screw-top glass vial and dissolved in 100 µL of 100% ethanol. Once dissolved, this ethanol/ vitamin E solution was added to a small volume of sterile 10% BSA in water and incubated overnight at 37°C. This solution of vitamin E conjugated to BSA was used to prepare various concentrations (0–25 µmol/L) of TRF-supplemented treatment media such that all control and treatment media had a final concentration of 5 mg/mL BSA. Ethanol was added to all treatment media such that the final ethanol concentration was the same in all treatment groups within a given experiment and was always less than 0.1%.

Prenoeplastic and neoplastic mammary epithelial cell number was determined in 24-well culture plates (6 wells/group) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colourimetric assay, as described previously. 13–15 On the day of assay, treatment media was replaced with fresh growth media containing 0.83 mg/mL MTT and the cells were returned to the incubator for 4 h. Afterwards, the media was removed and the MTT crystals were dissolved in 0.5 mL isopropanol. The optical density of each sample was read at 570 nm on a microplate reader (SpectraCount; Packard Bioscience, Meriden, CT, USA) against a blank prepared from cell-free cultures. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined using a haemocytometer, at the start of each experiment. 13–15 In separate control studies, various doses of TRF, 8-Br-cAMP, cholera toxin, pertussis toxin or forskolin were not found to affect the specific activity of the MTT colourimetric assay.

Whole cell lysates obtained from the different treatment groups were dissolved in Laemmlı buffer 16 and the protein concentrations of each sample were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s directions. Equal amounts of protein from each sample (25–50 µg/lane) in a given experiment were loaded on polyacrylamide minigels and electrophoresed through either a 12 or 7.5% resolving gel. Proteins were transblotted (25 V for 12–16 h) to polyvinylidene fluoride membranes (DuPont, Boston, MA, USA) according to the methods of Towbin et al. 17 Membranes were blocked with 2% BSA in 10 mmol/L Tris-HCl containing 50 mmol/L NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then incubated with either antiphosphotyrosine monoclonal...
antibody (Transduction Laboratories, Lexington, Ky, USA), anti-EGF receptor rabbit polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-Akt (or anti-active-Akt monoclonal antibody (Cell Signalling Technology, Beverly, MA, USA), anti-ERK1/ERK2 monoclonal antibody (Santa Cruz Biotech) or anti-active MAPK monoclonal antibody (Promega, Madison, WI, USA) in TBST with 2% BSA for 2 h. Membranes were rinsed five times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated goat antimouse or goat antirabbit secondary antibody (Pierce, Rockford, IL, USA) in TBST with 2% BSA for 1 h. Afterwards, blots were rinsed five times with TBST and protein bands were visualised by chemiluminescence according to the manufacture’s instructions (Pierce). In each experiment, blots from each treatment group were exposed on the same piece of X-OMAT AR film (Kodak, Rochester, NY, USA). Images were acquired with a Microtek 9600XL scanner (Redondo Beach, CA, USA) and analysed with Scion software.

Differences among the various treatment groups were determined by analysis of variance, followed by Duncan’s multiple range test. A difference of $P < 0.05$ was considered to be significant, as compared to controls or as defined in the figure legends.

**Results**

Preneoplastic CL-S1 cells grown in serum-free control media showed a four-fold increase in viable cell number over the duration of the 5-day culture period, whereas treatment with 5–20 $\mu$mol/L TRF significantly inhibited EGF-induced cell proliferation in a dose-responsive manner (Fig. 1). Because the mitogenic actions of EGF are mediated through specific membrane-bound tyrosine kinase receptors (EGF receptors), subsequent studies were conducted to investigate whether the antiproliferative effects of TRF on preneoplastic CL-S1 cells results from a reduction in EGF receptor levels and/or EGF-induced EGF receptor tyrosine kinase activity. Cells were grown for 5 days in media containing 0–25 $\mu$mol/L TRF. Cells within each treatment were then isolated by trypsin digestion, pooled, rinsed and whole cells lysates were then prepared for subsequent western blot analysis for EGF receptor and intracellular protein phosphotyrosine intensity. Results from these studies showed that treatment with various growth-inhibitory doses (5–25 $\mu$mol/L) of TRF had no effect on EGF receptor levels (Fig. 2) or EGF-induced EGF receptor-dependent intracellular protein phosphotyrosine intensity (Fig. 3) in preneoplastic CL-S1 mammary epithelial cells, as compared to controls. Scanning densitometric analysis of the EGF receptor protein bands showed little difference between treatment groups (Fig. 2).

Activation of the MAPK signalling cascade is an important downstream event associated with EGF-dependent mitogenesis in mammary epithelial cells. Studies were conducted to determine if TRF inhibition of EGF-dependent preneoplastic CL-S1 cell proliferation results from a reduction in ERK1 or ERK2 levels and/or activation. The effects of growth-inhibitory doses (5–10 $\mu$mol/L) of TRF on total ERK1 and ERK2 levels and phosphorylated (active) ERK1 and ERK2 levels as determined by western blot analysis for the EGF-receptor are shown in Figures 4 and 5, respectively. Total ERK1 and ERK2 levels in whole cell lysates obtained from CL-S1 cells maintained in control media showed intense bands visualised at 44 and 42 kDa, respectively (Fig. 4). Treatment with 5–10 $\mu$mol/L TRF resulted in a slight decrease in band intensity of ERK1 and ERK2 bands. Scanning densitometric analysis of the active ERK1 and ERK2 bands as well as the total active ERK bands is shown in Figures 4 and 5, respectively.
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However, these treatments were found to induce a dose-responsive decrease in both the 44 and 42 kDa band intensity of phosphorylated (active) ERK1 and ERK2, respectively, as compared to controls (Fig. 5). Scanning densitometric analysis of total (Fig. 4) and phosphorylated (Fig. 5) ERK1 and ERK2 are shown below their respective western blots.

Because the EGF receptor is a G-protein-coupled receptor in normal and neoplastic mammary epithelial cells, studies were conducted to determine if combined treatment of TRF with pharmacological agents that increase intracellular cAMP levels had any affect on TRF suppression of preneoplastic CL-S1 mammary epithelial cell proliferation. Dose–response studies were first conducted to determine the effects of various doses of cholera toxin (agent that stimulates Gs-proteins), pertussis toxin (agent that inhibits Gi-proteins), forskolin (agent that stimulates adenylyl cyclase) or 8-Br-cAMP (long-acting cAMP agonist) on EGF-dependent CL-S1 cell proliferation (data not shown). The highest treatment doses that had little or no effect on EGF-dependent CL-S1 mammary epithelial cell proliferation were then chosen for use in combination studies with TRF, as shown in Fig. 6. Treatment with 10 ng/mL cholera toxin, 5 ng/mL pertussis toxin, 20 µmol/L forskolin or 0.1 mmol/L 8-Br-cAMP alone had no affect or caused a slight decrease, whereas treatment with 5–10 µmol/L TRF alone significantly inhibited CL-S1 cell growth, as compared to controls (Fig. 6). However, combination treatment of these agents with 5–10 µmol/L TRF was found to completely reverse the growth-inhibitory effects of

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**Figure 3.** Effects of 0–25 µmol/L tocotrienol-rich palm oil fraction (TRF) on epidermal growth factor (EGF)-induced intracellular protein phosphotyrosine intensity in preneoplastic CL-S1 mammary epithelial cells grown for 5 days in culture. Whole cell lysates (25 µg/lane) from the different treatment groups were fractionated by SDS-PAGE and then subjected to western blot analysis for phosphotyrosine intensity.

**Figure 4.** Effects of growth inhibitory doses of tocotrienol-rich palm oil fraction (TRF) on total ERK1 and ERK2 levels in preneoplastic CL-S1 mammary epithelial cells grown in culture. Cells were grown in control media for 4 days and then divided into different groups, fed mitogen-free control or treatment media and returned to the incubator for 24 h. The next day all cells were treated with 10 ng/mL epidermal growth factor (EGF) for 10 min. (a) Whole cell lysates (50 µg/lane) from the different treatment groups were fractionated by SDS-PAGE and then subjected to western blot analysis for total intracellular ERK1 and ERK2 levels. (b) Scanning densitometric analysis of ERK1 and ERK2 bands are displayed below the western blot. Vertical bars represent the integrated optical density of bands visualised in each lane.

**Figure 5.** Effects of growth inhibitory doses (5 and 10 µmol/L) tocotrienol-rich palm oil fraction (TRF) on phosphorylated (active) ERK1 and ERK2 levels in preneoplastic CL-S1 mammary epithelial cells grown in culture. Cells were grown in control media for 4 days and then divided into different groups, fed mitogen-free control or treatment media and returned to the incubator for 24 h. The next day all cells were treated with 10 ng/mL epidermal growth factor (EGF) for 10 min. (a) Whole cell lysates (50 µg/lane) from the different treatment groups were fractionated by SDS-PAGE and then subjected to western blot analysis for phosphorylated (active) intracellular ERK1 and ERK2 levels. (b) Scanning densitometric analysis of ERK1 and ERK2 bands are displayed below the western blot. Vertical bars represent the integrated optical density of bands visualised in each lane.
TRF on EGF-dependent preneoplastic CL-S1 cell proliferation (Fig. 6).

Epidermal growth factor-induced elevations in intracellular cAMP levels are associated with the activation of cAMP-dependent protein kinases involved in mitogenic signalling, particularly the PI3K/Akt pathway. Therefore, studies were conducted to determine if the antiproliferative effects of TRF are mediated through a decrease in PI3K/Akt mitogenic signalling. Preneoplastic CL-S1 mammary epithelial cells were grown in control media for 4 days. Cells were then divided into different groups and treated with EGF-free control or treatment media for a 24-h period. The next day groups were treated with either vehicle or 10 ng/mL EGF. After a 10-min exposure to vehicle or EGF, whole cell lysates from each treatment group were prepared for subsequent western blot analysis for total Akt and phosphorylated (active) Akt. Figure 7 shows that the total intracellular levels of Akt levels were not affected by the presence or absence of EGF (controls) or treatment with TRF. However, exposure to EGF was found to cause an increase in the relative levels of phosphorylated (active) Akt, and treatment with 5 or 10 µmol/L TRF blocked this effect in preneoplastic CL-S1 mammary epithelial cells (Fig. 8). Scanning densitometric analysis of total (Fig. 7) and phosphorylated (active) Akt (Fig. 8) are shown below their respective western blots.

**Discussion**

The experimental results from the present study show that TRF inhibition of EGF-dependent preneoplastic CL-S1 mouse mammary epithelial cell growth does not result from a decrease in either EGF receptor levels or EGF-induced

![Figure 6](image-url)
EGF receptor tyrosine kinase activity. These data indicate that growth-inhibitory effects of tocotrienols occur down-stream from the EGF receptor in these cells. Previous studies have shown that the EGF receptor is a G-protein-coupled receptor and that EGF-induced activation of the EGF receptor results in an increase in intracellular cAMP levels and activation of cAMP-dependent protein kinases in both normal and neoplastic mammary epithelial cells.8–10 The present work reveals that combined treatment with pharmacological agents that either enhance G-protein function (cholera and pertussis toxin) or increase intracellular cAMP levels (forskolin and 8-Br-cAMP) completely reversed the growth-inhibitory effects of tocotrienols on preneoplastic CL-S1 cells. These findings indicate that the antiproliferative effects of tocotrienols result from an inhibition of G-protein-mediated activation of adenylyl cyclase and cAMP production. Additional studies showed that tocotrienols specifically inhibit cAMP-dependent protein kinase mitogenic signaling, particularly the PI3K/Akt pathway. These findings clearly identify the specific intracellular signalling pathways that are involved in mediating the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells, and provide new information that may be useful in developing effective therapeutic strategies for the prevention and/or treatment of breast cancer in women.

Although the composition of TRF is approximately 75% tocotrienols and 25% α-tocopherol, previous studies have demonstrated that antiproliferative effects of TRF are not mediated by α-tocopherol.2,3 Although tocopherols and tocotrienols have the same basic chemical structure, direct comparisons between the two vitamin E subclasses show that tocotrienols are significantly more potent than tocopherols and display a biopotency relationship corresponding to δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > δ-tocopherol > γ- and α-tocopherol.2,3 It is not completely understood why individual tocopherols and tocotrienols display differential potencies in regard to their antiproliferative activity in normal, preneoplastic and neoplastic mammary epithelial cells grown in culture. However, studies have also shown that these different cell types preferentially or selectively accumulate tocotrienols as compared to tocopherols.2,3 It has been suggested that because mammary epithelial cells preferentially take up tocotrienols over tocopherols, higher concentrations of tocotrienols are present at their intracellular
sites of action and are thereby able to induce a greater biological response. ²,³

Although the biological actions of EGF are mediated through specific membrane-bound EGF receptors, tocotrienol suppression of EGF-dependent mitogenesis was not found to be associated with a reduction in EGF-receptor mitogenic responsiveness. It is well established that activation of the EGF receptor can initiate multiple cAMP-dependent mitogenic signalling pathways, as shown in an abbreviated form in Figure 9. Activation by EGF of the G-protein-coupled EGF receptor will stimulate adenylyl cyclase, thereby elevating intracellular cAMP levels. ⁵-¹¹ Increased cAMP production will activate guanine nucleotide exchange factors (cAMP-GEF), such as Rap-Ras proteins that then activate Raf proteins, and subsequently lead to the activation of the MAPK mitogenic signalling cascade. ⁷,¹¹ EGFR receptor stimulation of cAMP-GEF and/or direct tyrosine phosphorylation of PI3K can also lead to the activation of the PI3K/Akt mitogenic pathway. ¹¹ Enzymatic activity of PI3K on cell membrane phospholipids generates 3′-phosphorylated phosphoinositides that bind and cause translocation of Akt to the cell membrane. ¹⁸,¹⁹ Akt is then capable of being phosphorylated to its active state by membrane phosphoinositide-dependent serine/threonine regulatory kinases. ¹¹,¹⁸,¹⁹ Activation of Akt subsequently leads to the phosphorylation and regulation of various targets involved in mitogenesis, including transcription factors such as forkhead and Daf 12. ¹¹,¹⁸,¹⁹ Activation of protein kinase A by elevated intracellular cAMP is another important mitogenic pathway in various cell types, ¹⁸,¹⁹ but additional studies are required to determine the role of tocotrienols in modulating this cAMP-dependent signalling pathway in preneoplastic mammary epithelial cells.

Although treatment with growth-inhibitory doses of TRF was observed to inhibit both MAPK and Akt activation, combined treatment with pharmacological agents that increase intracellular cAMP levels could reverse the antiproliferative effects of TRF. These findings strongly suggest that tocotrienols indirectly attenuate EGF-dependent MAPK and PI3K/Akt mitogenic signalling by inhibiting early post-EGF receptor upstream events involved in cAMP production. At present, the exact intracellular site of action involved in mediating the growth-inhibitory effects of tocotrienols is unknown. However, results from the present study suggest that tocotrienols most likely act to inhibit EGF-induced EGF receptor G-proteins and/or adenylyl cyclase activation in preneoplastic mammary epithelial cells. Additional studies are needed to determine if the antiproliferative effects of tocotrienols in normal and neoplastic mammary epithelial cells are mediated through similar cAMP-dependent mechanisms as was observed in preneoplastic cells.

In summary, the present findings show that the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells do not result from a reduction in EGF receptor mitogenic responsiveness, but from the inhibition of early post-receptor downstream events associated with cAMP production and cAMP-dependent kinase signalling. Further characterisation of the intracellular mechanisms of action of tocotrienols will elucidate the potential value of these vitamin E compounds as chemopreventive agents, either alone or in combination with traditional therapies in the treatment of breast cancer.

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![Figure 9. A generalised schematic representation of EGF-induced cAMP-dependent mitogenic signalling pathways in preneoplastic CL-S1 mammary epithelial cells. cAMP, cyclic AMP; EGF, epidermal growth factor; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated pathway kinase; PDK, P13K dependent kinase; PI3K, phosphoinositide 3-kinase.](image-url)


