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Differential Suppression of Proliferation in MCF-7 and MDA-MB-231 Breast Cancer Cells Exposed to α-, γ- and δ-Tocotrienols Is Accompanied by Altered Expression of Oxidative Stress Modulatory Enzymes

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Abstract. *Tocotrienols belong to the vitamin E family of chemicals known to have potent anti-proliferative and apoptotic activities against a variety of cancer cells with little to no comparable influence on the normal cells. Whether tocotrienols control the expression of phase II antioxidant enzymes in the context of their anti-carcinogenic mechanisms has not been investigated. The present studies were performed to test whether the differential growth inhibition resulting from exposure to α-, γ- and δ-tocotrienols in estrogen receptor-positive human MCF-7 and estrogen receptor-negative MDA-MB-231 breast cancer cells might be accompanied by changes in phase II antioxidant enzymes. Cell proliferation and clonogenicity in both cell lines were significantly inhibited by γ- and δ-tocotrienols with little affect when cells were similarly exposed to α-tocotrienol, at doses up to 10 μM. The expression and activity of several antioxidant enzymes in 10 μM tocotrienol-treated cells were determined by Western blot and biochemical assays. In MDA-MB-231 cells, δ- was more active than α- or γ-tocotrienols in up-regulating glutathione peroxidase; however, the three tocotrienols had comparable activity in inducing thioredoxin. In MCF-7 cells, expression of quinone reductase 2 and thioredoxin was increased by γ- and δ-tocotrienols, whereas quinone reductase 1 was unaffected by exposure to the tocotrienols. The tocotrienols also did not affect the expression and activity of superoxide dismutase in both MCF-7 and MDA-MB-231 cells, but increased catalase activity concomitant with slight reduction in the catalase expression. In MDA-MB-231 cells, treatment by tocotrienols led to several fold increase of NRF2 expression marked by corresponding decrease in KEAP1 levels. By contrast, no significant change in NRF2 and KEAP1 levels was observed in MCF-7 cells. These studies demonstrate that different tocotrienols show distinct and selective activity in regulating the NRF2-KEAP1, in coordination with the induced expression of cytoprotective oxidative stress modulatory genes and regulation of proliferation in breast cancer cells.*

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Breast cancer is a devastating disease that affects tens of thousands of American women each year. Although a definitive predictor for breast cancer development is not available, a number of factors have been identified that facilitate the classification of women with predisposed risk to the disease (1-3). Breast cancer risk factors have provided a multidisciplinary framework and strategy encompassing prevention, diagnosis, and therapy to more effectively and significantly reduce the incidence and mortality of breast cancer (4-6). Included in the envisaged approaches for breast cancer prevention is the use of micronutrients whose efficacy is supported by *in vitro* studies, animal and human experiments, and data from intervention clinical trials (7-9).

The vitamin E family is known for its cellular antioxidant and lipid lowering properties (10-14). It comprises tocopherals and tocotrienols which differ structurally with respect to the chemical nature of the phytyl chain resulting in the existence of four isomeric forms, respectively, alpha (α), beta (β), gamma (γ), and delta (δ), based on the location of the methyl group on the aromatic ring (15, 16). Tocotrienols possess antioxidant, anti-inflammatory, anti-angiogenic, and anti-proliferative properties (15, 17-21). Accumulating evidence also suggests that tocotrienols display chemopreventive attributes (18, 19, 22-24). For example, tocotrienols and tocotrienol-rich fractions from palm oil have been shown to inhibit the growth of human breast cancer cells (25-27). Consumption of palm oil in the diet, in contrast to the

intake of other high-fat diets, suppresses carcinogen-induced tumorigenesis in the mammary gland of experimental animals (28-31). Dietary supplementation of tocotrienol-rich fraction has been shown to inhibit growth of estrogen receptor-positive human breast cancer MCF-7 cells in athymic nude mice (32, 33).

The potential health benefits of tocotrienols in reducing the risk of breast cancer in women have not been fully explored, owing in part to a paucity of information regarding mechanisms underlying their chemopreventive effects. Since phase II antioxidant enzymes may play an important role in the anti-carcinogenic mechanisms of tocotrienols, the present studies were undertaken to explore the relationship between the differential growth inhibition resulting from exposure of estrogen receptor-positive human MCF-7 and estrogen receptor-negative MDA-MB-231 breast cancer cells to α-, γ- and δ-tocotrienols and accompanying changes in their antioxidant enzyme defense response.

Materials and Methods

Reagents. α-, γ- and δ-tocotrienols were purchased from Cayman chemicals (Ann Arbor, MI, USA). Bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione, menadione, dicoumarol, NADPH, glutathione reductase (GR) and anti-superoxide dismutase (SOD-1) antibody were purchased from Sigma Chemicals (St. Louis, MO, USA). Primary antibodies including anti-glutathione peroxidase (GPX), anti-thioredoxin (TRX), anti-quinone reductase 1 (NQO1), anti-quinone reductase 2 (NQO2) anti-KEAP1, anti-NRF2, anti-actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-catalase antibody was purchased from Calbiochem Inc. (San Diego, CA, USA). Fetal bovine serum (FBS), L-15 and MEM (Minimum Essential Medium Eagle) media, trypsin, penicillin and streptomycin were purchased from Cellgro, Inc. (Herndon, VA, USA). Acrylamide and *bis*-acrylamide were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other chemicals and solvents used were of analytical grade.

Cell culture and treatment. Human MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA) and maintained in MEM and L-15 media, respectively, supplemented with penicillin, streptomycin and 10% heat inactivated FBS in a humidified atmosphere at 37˚C. The MCF-7 cells were maintained in an atmosphere of 5% CO_2/95% air and MDA-MB-231 cells were maintained without CO_2 as previously described (34). α- And δ-tocotrienols were dissolved in ethanol and added to the culture media to the final concentration specified in the text. γ-Tocotrienol was dissolved in dimethyl sulfoxide (DMSO). The final concentration of ethanol or DMSO in culture medium during treatment did not exceed 0.1% (v/v), and the same concentration of ethanol or DMSO was added to the control dishes.

Cell growth assay. MCF-7 and MDA-MB-231 cells were plated at 5×10^4 cells/ml in 6-well plates under standard culture conditions. After 24 h, cells were treated with either vehicle alone or different doses of tocotrienols. After 72 h of treatments, cells were trypsinized, collected, and counted using a hemocytometer.

Colony formation assay. Colony formation assay was performed as described previously with some modifications (34). MCF-7 and MDA-MB-231 cells were plated in 6-well tissue culture plates at a density of 800 cells/ml, 2 ml/well. After 24 h, cells were treated with either vehicle alone or different doses of tocotrienols, followed by an additional 8-day incubation to allow colonies to form. Colonies were fixed and stained with 1.25% crystal violet, washed to remove excessive dye, and imaged by a scanner. Quantitative changes in clonogenicity were determined by extracting colonies with 10% acetic acid and measuring the absorbance of the extracted dye at 595 nm.

Preparation of cell extracts. For Western blotting, cells were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail). Protein content of cell lysates were determined with a Coomassie protein assay kit (Pierce, IL, USA) with BSA as standard. To measure the activities of enzymes, cells were sonicated in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA, followed by centrifugation at 13,000 × *g* for 10 min at 4˚C. The supernatants were collected for the immediate measurement of the activities of enzymes. Protein contents of cell lysates were determined as described above.

Immunoblotting. The proteins (20 μg of protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a nitrocellulose membrane (Whatman, Middlesex, UK) and then blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween 20) containing 3% nonfat dried milk. The blots were probed with primary antibodies as specified, followed by secondary antibodies. The blots were then developed and visualized with the enhanced chemiluminescent (ECL) detection system (KPL, Gaithersburg, MD, USA). In some instances blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol and 2% SDS) for 5 min at 50˚C and then reprobed with other antibodies. The intensity of the specific immunoreactive bands were quantified by densitometry and expressed as a ratio to the internal control.

Assay of superoxide dismutase (SOD). SOD activity was assayed using a commercially available kit (Fluka, Buchs, Switzerland) which utilizes a highly water soluble tetrazolium salt, WST (2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water soluble dye upon reduction with superoxide anion. The rate of inhibition of the activity of xanthine oxidase by SOD was measured and expressed as the enzyme required for 50% inhibition of xanthine oxidase activity/min/mg protein.

Assay of catalase. Catalase was assayed by the method of Aebi (35). Briefly, the reaction was started by adding H_2O_2 into cell lysate in potassium phosphate buffer (pH 7.0) and the decomposition of H_2O_2 was monitored at 25˚C for 1 min. The extinction coefficient (0.0436 $mM^{-1}cm^{-1}$) was used to calculate the catalase activity, expressed as μmol of H_2O_2 decomposed/min/mg protein.

Assay of quinone reductase 1 (NQO1) activity. Activity of NQO1 was assayed by the method of Lind *et al*. (36) with slight modifications. Briefly, the reaction was initiated with the addition

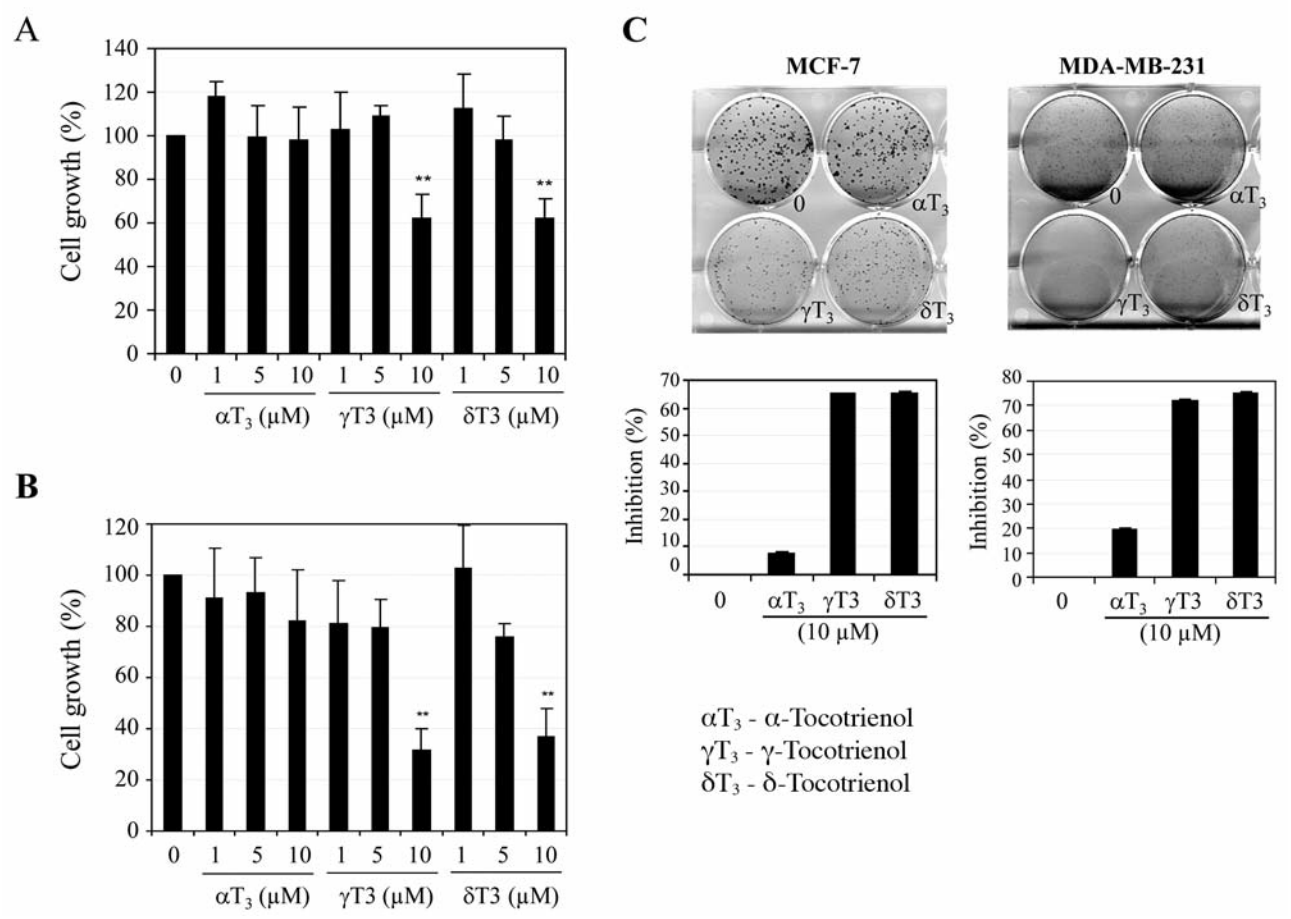

Figure 1. *Effect of tocotrienols on MCF-7 and MDA-MB-231 cell proliferation and colony formation. A: MCF-7 cells were treated with increasing doses (1, 5, 10 μM) of α-, γ- and δ-tocotrienol and the cell numbers were counted at 72 h using a hemocytometer. The cell growth was expressed relative to control. Values are expressed as mean±SD for three experiments. **Statistical significance of p<0.001, when compared to vehicle-treated control. B: MDA-MB-231 cells were treated with increasing doses (1, 5, 10 μM) of α-, γ- and δ-tocotrienol for 72 h. The cell growth was expressed relative to control. Values are expressed as mean±SD for three experiments. **Statistical significance of p<0.001, when compared to vehicle-treated control. C: MCF-7 or MDA-MB-231 cells were plated, in triplicate, in 6-well tissue culture plates at a density of 800 cells/ml, in the absence or presence of the indicated dose of tocotrienols. On the day of cell harvest, colonies were stained with 1.25% crystal violet, and then extracted with 10% acetic acid. The acetic acid extract was quantified by measuring the absorbance at 595 nm.*

of menadione into the reaction mixture containing 33 mM potassium phosphate buffer (pH 7.4), 0.18 mM NADPH, 0.02% BSA, 0.01% Tween-20 and cell lysates. The oxidation of NADPH was followed spectrophotometrically at 340 nm, 25˚C for 2 min with and without 20 mM dicoumarol. The dicoumarol-sensitive part was taken as NQO1 activity and an extinction coefficient (6.22 $mM^{-1}cm^{-1}$) was used to calculate NQO1 activity, expressed as μmol of NADPH oxidized/min/mg protein.

Assay of glutathione peroxidase (GPX) activity. Cellular GPX activity was measured by the method of Flohe and Gunzler (37). Briefly, the reaction mixture contained 50 mM potassium phosphate (pH 7.0), 10 mM GSH, GR (2.4 U/ml), 1.5 mM NADPH and cell lysate. The mixture was incubated at 37˚C for 3 min. After the addition of 100 μl of 2 mM H_2O_2, the rate of NADPH oxidation was monitored at 340 nm for 5 min. The nonspecific oxidation of NADPH was corrected by replacing the cell lysate with assay buffer. GPX activity was calculated and expressed as μmol of NADPH oxidized/min/mg protein.

Data analysis. The results are expressed as mean±standard deviation (SD). Differences between groups were assessed by one-way analysis of variance using the SPSS software package for Windows, Version 14.0 (Chicago, IL, USA). *Post-hoc* testing was performed for inter-group comparisons using the least significance difference (LSD) test; statistical significance at *p*-values <0.001 or <0.01 were presented using respective symbols in the figure legends.

Results

Tocotrienols inhibit MCF-7 and MDA-MB-231 breast cancer cell growth. We first tested the ability of α, γ and δ-tocotrienols to inhibit growth of the MCF-7 and MDA-MB-

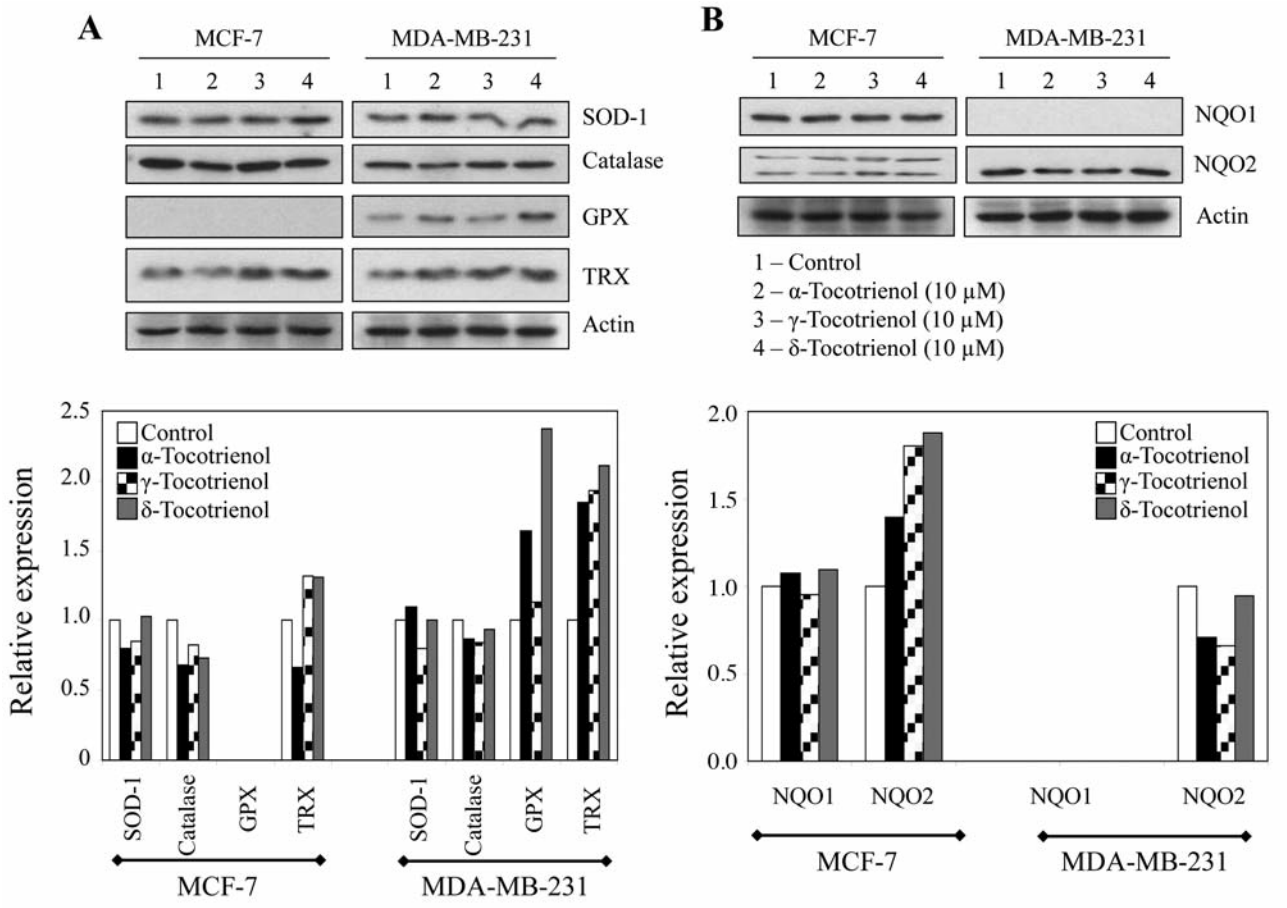

Figure 2. *Effect of tocotrienols on the expression of antioxidant and phase II detoxification enzyme NQO1 and NQO2 in MCF-7 and MDA-MB-231 cells. Cells were exposed to tocotrienols at 10 μM concentration for 72 h. Cell lysates were prepared and protein was resolved by SDS PAGE and subjected to Western blot analysis. Densitometric analysis was performed and the expression of the indicated proteins was expressed relative to controls.*

231 breast cancer cell lines (Figure 1A and 1B). Comparing the effects of α-, γ-, and δ-tocotrienols on breast cancer cell proliferation, we observed that γ- and δ-tocotrienols significantly inhibited growth in both cell lines at 10 μM concentration. Since the 10 μM dose consistently elicited strong suppression of cell proliferation, this concentration was used to further explore affects of tocotrienols on the clonogenicity of MCF-7 and MDA-MB-231 cells. Results in Figure 1C showed that the inhibition of colony formation occurred in the following fashion in both cell lines: δ-tocotrienol≥γ-tocotrienol>α-tocotrienol.

Tocotrienols alter the expression of antioxidant and detoxification enzymes. Oxidative stress is considered to be a contributing factor for cancer initiation and development; conceivably, chemopreventive agents might affect the different stages of carcinogenesis by counteracting oxidative stress. In the case of tocotrienols, although they are known to act as antioxidants, the possibility that they also utilize other mechanisms to affect intracellular redox status has not been systematically investigated. Accordingly, the expression of antioxidant defense proteins was assessed by western blot analysis. In MDA-MB-231 cells, all three tocotrienols comparably induced the expression of TRX but had no effect on the levels of SOD-1 and catalase (Figure 2A). By comparison, the induction of GPX expression in these cells was dependent on the tocotrienols used, the relative potency being δ-tocotrienol>α-tocotrienol>γ-tocotrienol (Figure 2A). In the case of MCF-7 cells, different results were obtained. First, no immunoreactive GPX was detected suggesting that this enzyme is present in extremely low levels of expression. A moderate increase in the TRX was observed in MCF-7 cells exposed to δ- and γ-tocotrienol, whereas α-tocotrienol caused a slight reduction in TRX (Figure 2A). Moreover, all three tocotrienols reduced the expression of catalase but had no noticeable effect on SOD-1 levels (Figure 2A).

We also analyzed the changes in expression of phase II detoxification enzymes NQO1 and NQO2 exposed to α-, γ-

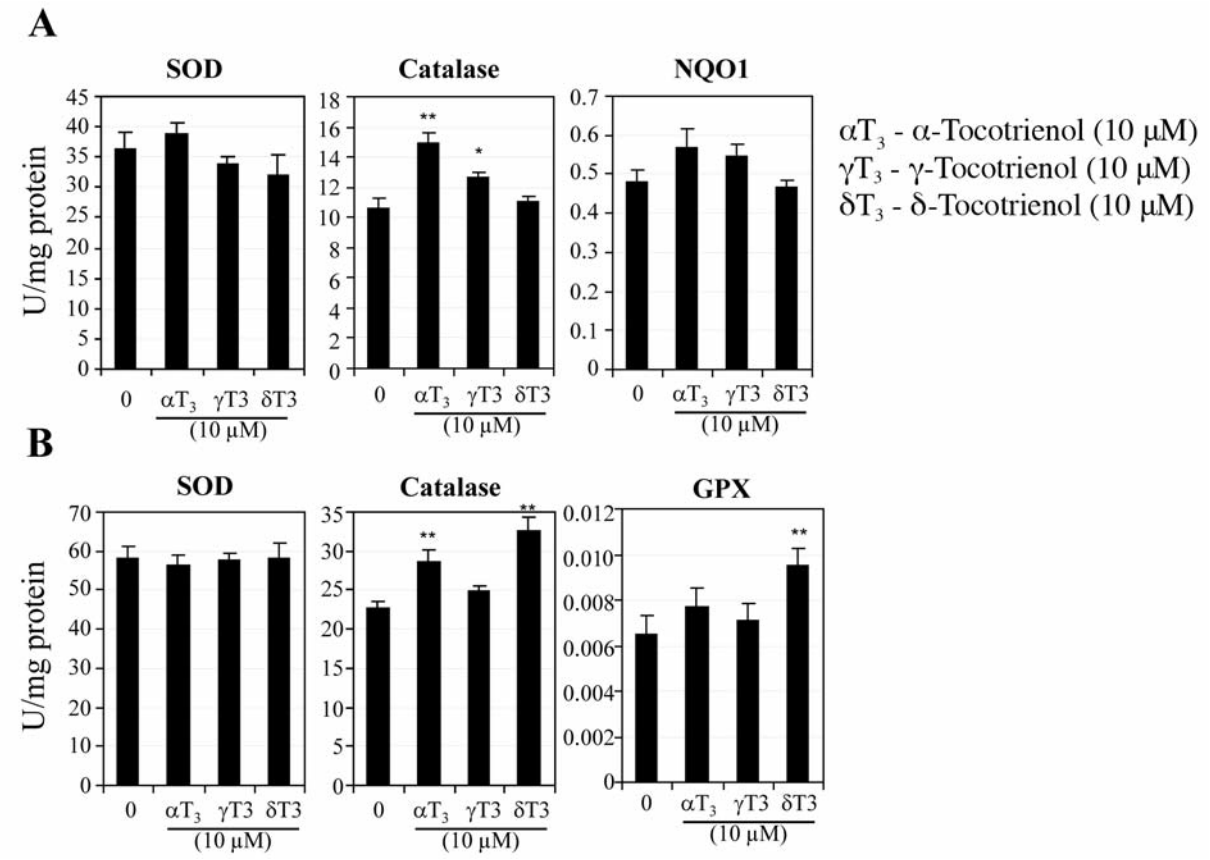

Figure 3. *Tocotrienol-induced alterations on the activities of antioxidant enzymes in MCF-7 (A) and MDA-MB-231 (B) cells. MCF-7 cells were incubated with the tocotrienols (10 μM) and the activities of antioxidant enzymes were determined. Values are mean±SD for three experiments. Enzyme activities are expressed as follows: SOD: amount of enzyme that inhibits the activity of xanthine oxidase by 50%; Catalase: μmol of H_2O_2 decomposed/min; NQO1: μmol of NADPH oxidized/min. *p<0.01 and **p<0.001, as compared to control.*

and δ-tocotrienols (Figure 2B). In MCF-7 cells, the expression of NQO2 was significantly induced by γ- and δ-tocotrienol, but the expression of NQO1 was not altered by tocotrienols. In contrast to MCF-7, α- and γ-tocotrienol decreased the expression of NQO2 in MDA-MB-231 cells; notably, these cells had undetectable NQO1 levels (Figure 2B).

Changes in antioxidant and detoxification enzyme activities in tocotrienol-exposed cells. The alterations in the expression of antioxidant enzymes in response to treatment by tocotrienols were further assessed by measuring changes in the activity of the respective enzymes. In MCF-7 cells exposed to α- or γ-tocotrienol, catalase activity showed a modest increase while the activity of SOD and NQO1 was not altered (Figure 3A). In MDA-MB-231 cells, treatment by α- or δ-tocotrienol was accompanied by a significant increase in catalase activity; δ-tocotrienol also increased GPX activity, while SOD activity was not affected by tocotrienols (Figure 3B).

Tocotrienols induces NRF2/KEAP1 pathway. Since tocotrienols alter the expression and activities of some antioxidant and detoxification enzymes, and because their regulation is at least in part under the NRF2/KEAP1 transcription control (38-43), we next measured the effects of tocotrienols on NRF2 and KEAP1 expression by Western blot analysis. In MDA-MB-231 cells, treatment with α-, γ- and δ-tocotrienol resulted in 2.6-, 1.9- and 3.2-fold increase, respectively, in NRF2 protein levels, with a corresponding decrease in KEAP1 expression. In MCF-7 cells, no significant changes in NRF2 and KEAP1 levels were detected (Figure 4).

Discussion

The anticancer activities of tocotrienols have been shown in several studies previously (15, 17, 22, 23). However, reports on the cancer chemopreventive properties of tocotrienols are still quite limited and its role in NRF2 and antioxidant gene expression has never been investigated. The results of this

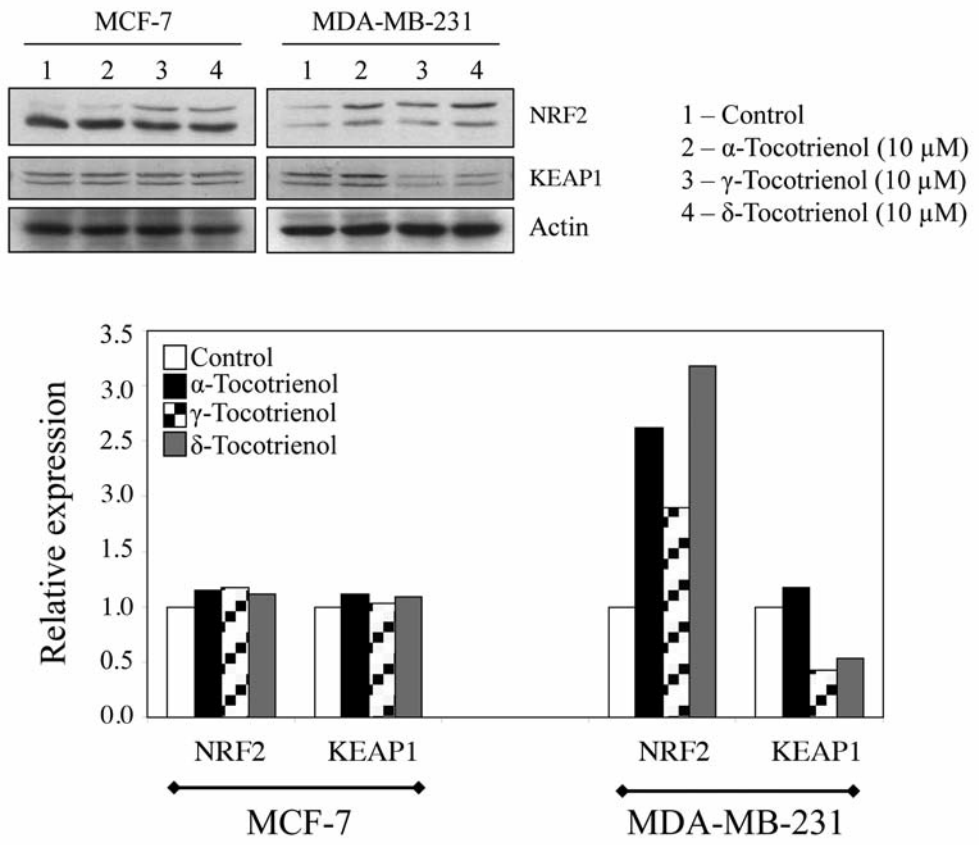

Figure 4. *Effect of tocotrienols on the expression of NRF2-KEAP1 in MCF-7 and MDA-MB-231 cells. Cells were exposed to tocotrienols at 10 μM for 72 h. Cell lysates were prepared and protein was resolved by SDS-PAGE and subjected to Western blot analysis. Densitometric analysis was performed and the expression of the indicated proteins was expressed relative to controls.*

study provide new evidence for a chemopreventive potential of tocotrienols, revealing three novel observations: (i) γ- and δ-tocotrienols were significantly more inhibitory than α-tocotrienol in affecting both MCF-7 and MDA-MB-231 cell proliferation, (ii) tocotrienols altered the redox status of MCF-7 and MDA-MB-231 cells, (iii) tocotrienols selectively induced the expression of NRF2 concomitant with a decrease in KEAP1 expression in MDA-MB-231 but not in MCF-7 cells.

Previous studies have shown that γ- and δ-tocotrienols control breast cancer cell proliferation by a mechanism independent of the estrogen receptor status (44); a similar conclusion was reached in this study regarding cells exposed to α-tocotrienol despite its less potent growth inhibitory effect. Moreover, this study also demonstrated that tocotrienol selectively and significantly increased the basal levels of antioxidative proteins, *e.g.* TRX only in MDA-MB-231 but not in MCF-7 cells. Interestingly, basal expression of GPX was only detected in MDA-MB-231 cells and further increases in GPX levels were elicited upon exposure of these cells to δ-tocotrienol. In contrast, the expression and activity of SOD in both cell types was not altered by tocotrienols; whereas tocotrienols caused a slight increase in catalase activity in both cell lines. It is noteworthy that GPX and catalase are both considered important cellular defenses in the decomposition of H_2O_2 (45-47); accordingly, their modulation by the tocotrienols could further tip the finely and dynamically controlled antioxidant system so as to favor the maintenance of a redox cellular state which could constitute part of the driving force underlying the chemopreventive potential of the tocotrienols.

Another significant finding of this study is the demonstration of the involvement of the NRF2/KEAP1 pathway in tocotrienol-mediated induction of antioxidant and detoxification enzymes in MDA-MB-231 cells. Our observation that tocotrienols decreased the level of KEAP1 and increased the accumulation of NRF2 may be an integral intermediate step involved in some of the increased expression of phase II and antioxidative enzymes. The mechanism(s) by which exposure to tocotrienols activate the NRF2/KEAP1 regulatory complex remains to be elucidated but conceivably may involve the modification of either of these proteins, by

indirect or direct mechanisms. A distinct possibility lies in post-translational modification (phosphorylation) events targeting this complex by various protein kinase signaling pathways, *e.g.* protein kinase C, mitogen-activated protein kinase (MAPK) cascades and phosphatidylinositol 3-kinase, which could regulate the decoupling of NRF2 from KEAP1, followed by its subsequent translocation into the nucleus (7).

In conclusion, the studies reported in this communication demonstrate for the first time the ability of tocotrienols to selectively regulate the NRF2-KEAP1 system, as a possible molecular antecedent for the induced expression of cytoprotective genes. Activation of this NRF2 signaling pathway may contribute to the beneficial effects of this micronutrient as an integral step in the prevention and therapy of breast cancer.

Acknowledgements

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