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Antioxidants in Dietary Oils: Their Potential Role in Breast Cancer Prevention

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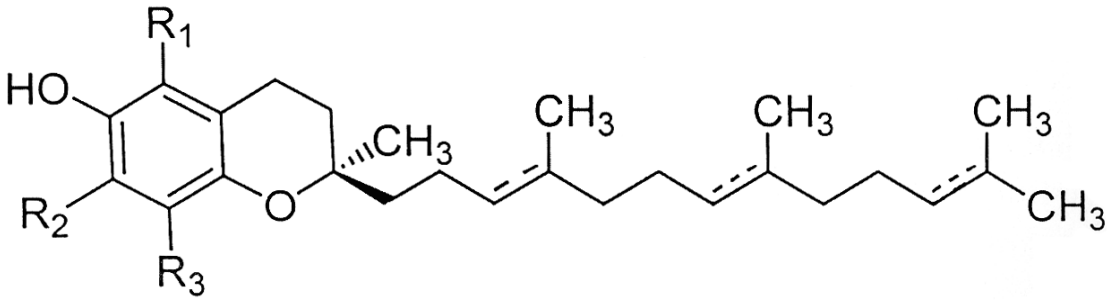
ABSTRACT

Edible oils contain variable amounts of natural antioxidants such as vitamin E. Antioxidants act not only to prevent lipid peroxidation and free-radical production, but also display potent anticancer activity. The vitamin E family of compounds is divided into two subgroups called tocopherols and tocotrienols, but only tocotrienols display potent anticancer activity at treatment doses that have little or no effect on normal cell growth or viability. Palm oil contains the highest concentrations of natural tocotrienols. Tocotrienols induced apoptosis or programmed cell death in breast cancer cells. Morphological and biochemical characteristics of apoptosis, such as nuclear and cytoplasmic condensation and DNA fragmentation, are mediated by the activation of cysteine proteases called caspases. Apoptosis is triggered by the activation of initiator caspases (caspase-8 or 9) that subsequently activate effector caspases (caspase-3, 6, and 7). Studies were conducted using the highly malignant +SA mouse mammary epithelial cell line to determine if tocotrienol-induced programmed cell death is mediated through the caspase-8 or caspase-9 pathway. Treatment with cytotoxic doses of tocotrienol resulted in a large increase in caspase-8 and caspase-3, but not caspase-9 activity. Combined treatment of tocotrienol with selective caspase-8 or caspase-3 inhibitors completely blocked tocotrieno-linduced apoptosis and activation of caspase-8 and caspase-3, respectively. These findings demonstrate that tocotrienol-induced apoptosis in highly malignant mammary epithelial cells is mediated through caspase-8 activation, and may provide essential information necessary for understanding the potential health benefits of these compounds in preventing and/or reducing the risk of breast cancer in women.

INTRODUCTION

Dietary fats and oils contain variable amounts of natural antioxidants that act to prevent spoilage, as well as maintain flavor and nutritional value. However, these natural antioxidants have also been shown to provide significant health benefits. Antioxidants act to inhibit the damaging affects of peroxidation reactions and free radical production within the body (Packer, 1991; Elson, 1992; Kimmick, Bell & Bostick, 1997). Uncontrolled production of free radicals is associated with damage to cell structures, reduced cellular function, and implicated as a cause of various diseases, such as arteriosclerosis and cancer (Packer, 1991; Elson, 1992; Kimmick *et al.*, 1997). Red palm oil is unique as compared to other dietary fats in that palm oil contains the highest known concentrations of natural antioxidants, particularly provitamins A carotenes and vitamin E (Khor & Raajeswari 2001). Although both vitamins A and E contribute to the maintenance of good health and disease prevention, palm vitamin E is of particular interest because it is composed of 70-80% tocotrienols (McIntyre *et al.*, 2000a). Vitamin E represents a

family of compounds that is further divided into two subclasses called tocopherols and tocotrienols. Tocopherols and tocotrienols have the same basic chemical structure characterized by a long phytyl tail attached to a chromane ring. However, tocopherols have a saturated, whereas tocotrienols have an unsaturated phytyl tail, and individual isoforms of tocopherols and tocotrienols differ from each other based on the degree of methylation of the chromane ring (Figure 1). Previous investigations have demonstrated that tocotrienols, but not tocopherols display potent antiproliferative and apoptotic activity against breast cancer cells (McIntyre *et al.*, 2000a; McIntyre *et al.*, 2000b; Sylvester *et al.*, 2001). Furthermore, the anticancer effects of tocotrienols were observed using treatment doses that had little or no effect on normal mammary epithelial cells growth or viability (McIntyre *et al.*, 2000a; McIntyre *et al.*, 2000b; Sylvester *et al.*, 2001). Although the exact reason why tocotrienols are more potent antitumor agents than tocopherols is presently unknown, these findings suggest that tocotrienols may have significant value as therapeutic agents for breast cancer prevention and/or treatment.



Compound	R_1	R_2	R_3	Phytyl Chain
α-tocopherol	CH_3	CH_3	CH_3	Saturated
γ-tocopherol	H	CH_3	CH_3	Saturated
δ-tocopherol	H	H	CH_3	Saturated
α-tocotrienol	CH_3	CH_3	CH_3	Unsaturated
γ-tocotrienol	H	CH_3	CH_3	Unsaturated
δ-tocotrienol	H	H	CH_3	Unsaturated

Figure 1. Generalized structure of vitamin E compounds

At present, the exact intracellular mechanism(s) mediating tocotrienol-induced programmed cell death or apoptosis in breast cancer cells is unknown. However, apoptosis is an important aspect of normal mammary gland growth and remodeling, as well as a mechanism by which neoplastic cells can be eliminated from the breast (Hockenbery *et al.*, 1990; Oltvai & Korsmeyer 1994; Heermeier *et al.*, 1996; Nass *et al.*, 1996). Morphological and biochemical characteristics distinguish apoptosis from necrosis in terms of nuclear and cytoplasmic condensation, DNA fragmentation, dilation of the endoplasmic reticulum, and alterations in the cell membrane composition (Hockenbery *et al.*, 1990; Oltvai & Korsmeyer, 1994; Heermeier *et al.*, 1996; Nass *et al.*, 1996). Initiation of cell death involves the activation of cysteine proteases known as caspases (Cohen 1997; Nicholson 1999). Initiator caspases (caspases-8, and -9) activate effector caspases (caspases-3, -6 and -7), which cleave structural and regulatory proteins (DFF45/ICAD, PARP, lamins, cytokeratins, etc) and are responsible for most features described above (Cohen 1997; Muzio *et al.*, 1998; Srinivasula *et al.*, 1998; Nicholson 1999).

There are at least two general mechanisms involved in caspase activation. Receptor-mediated caspase activation and apoptosis involves the activation of "death receptors", members of the tumor necrosis factor (TNF) receptor superfamily, by their corresponding ligands. Receptor activation results in trimerization and recruitment of adapter proteins through homophilic DD interactions, forming a complex that directly binds and promotes caspases-8 activation, leading to caspases -3 and/or -7 activation, and ultimately apoptosis (Ashkenazi & Dixit 1998). In contrast, mitochondrial stress-induced caspase activation and apoptosis can be initiated by numerous cellular signals that cause perturbations in the mitochondria resulting in the release of proapoptotic molecules such as AIF and cytochrome c from the intermembrane space into the cytoplasm (Green & Reed 1998; Susin *et al.,* 1999). Cytochrome c then interacts with Apaf-1, dATP/ATP and procaspase-9 to form a complex (apoptosome), which induces the activation of initiator caspase-9, and leads to the activation of effector caspase-3 and/or -7 and apoptosis (Li *et al.,* 1997; Zou *et al.,* 1997; Li *et al.,* 1998). Cytochrome c release from the mitochondria is required for apoptosome formation and is tightly controlled by the Bcl-2 family of proteins that exist to inhibit (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1) or promote (Bax, Bid, Bak, Bok, Bik, Hrk, Bim and Bad) apoptosis through the regulation of cytochrome c release (Oltvai & Korsmeyer 1994). It has also been shown that cross talk between the receptor- and mitochondrial stress-mediated pathways can occur. Studies have shown that activation of caspase-8 or downstream effector caspases lead to the release of cytochrome c from the mitochondria and caspase-9 activation in some cells (Li *et al.* 1998; Desagher *et al.,* 1999).

Various signaling pathways have been suggested for mediating vitamin E-dependent apoptosis, and tocopherol-and tocotrienol-induced apoptosis appears to be mediated by different mechanisms in different cell types. Studies were conducted to determine whether death receptor or mitochondrial stress-mediated signaling pathways are involved in tocotrienol-induced apoptosis.

MATERIALS AND METHODS

Experimental procedures have been previously described in detail (Sylvester *et al.,* 1994; McIntyre *et al.* 2000a; McIntyre *et al.,* 2000b; Sylvester *et al.,* 2001). The highly malignant +SA mammary epithelial cell line was derived from an adeno-carcinoma that developed spontaneously in a BALB/c female mouse (Danielson, Anderson & Hosick, 1980; Anderson, Danielson & Hosick, 1981). When injected back into the mammary gland fat pad of syngeneic female mice, the +SA cells form anaplastic adenocarcinomas and metastasize to the lung (Danielson *et al.,* 1980; Anderson *et al.,* 1981). The +SA cells can be grown on plastic and display rapid anchorage-independent growth when cultured in soft agarose gels (Danielson *et al.,* 1980; Anderson *et al.,* 1981). +SA cells were maintained in serum-free control media consisting of DMEM/F12 containing 5mg/ml bovine serum albumin (BSA), 10mg/ml transferrin, 100U/ml soybean trypsin inhibitor, l00U/ml penicillin, 0.1mg/ml streptomycin, 10ng/ml EGF, and 10μg/ml insulin (McIntyre 2000a), in the presence or absence of 1μM of specific caspase inhibitors (Calbiochem-Novabiochem Corporation, La Jolla, CA). Cells were divided into different treatment groups and fed serum-free control or treatment media. In order to dissolve the highly lipophilic vitamin E compounds in aqueous culture media, these compounds were first conjugated to bovine serum albumin (Sylvester *et al.,* 2001). The palm vitamin E extract, also

called the tocotrienol-rich-fraction of palm oil (TRF), was assayed by HPLC prior to use in experimentation, and determined to have a composition 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 lipid-soluble contaminant. Viable +SA mammary epithelial cell number was determined using the 3-(4,5-dimethylthiazol-2y1) -2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Sylvester *et al.,* 1994; Sylvester *et al.,* 2001). Treatment-induced apoptosis was determined by DNA fragmentation using phenol/chloroform extraction and TUNEL immunocytochemical assay (McIntyre *et al.,* 2000a; McIntyre *et al.,* 2000b). Measurement of treatment effects on caspase levels enzymatic activity was determined using Western blot analysis and enzyme activity colorimetric assay kits, respectively.

RESULTS

The effects of various doses of TRF on +SA cell proliferation are shown in Figure 2. Neoplastic +SA cells grown in serum-free control media displayed a continuous increase in viable cell number over the 5-day culture period (Figure 2). Culture medium supplemented with 2-8 μM TRF significantly inhibited EGF-induced cell proliferation in a dose-responsive manner, as compared to controls (Figure 2).

Since TRF contains a number of vitamin E isoforms, it was not possible to determine if one or all of these isoforms were responsible for mediating inhibitory effects of TRF on +SA cell growth described in Figure 2. Therefore, additional studies were conducted to determine the antiproliferative effects of specific tocopherol and tocotrienol isoforms on neoplastic +SA mammary epithelial cell. After 5 days of culture, treatment with 0-120 μM α- or γ-tocopherol had no effect, whereas treatment with 30-120 μM δ-tocopherol significantly inhibited cell growth, as compared to controls (Figure 3). Treatment with 4-30 μM α-tocotrienol, or 3-10 μM γ- or δ-tocotrienol significantly inhibited +SA cell growth, compared to controls (Figure 3).

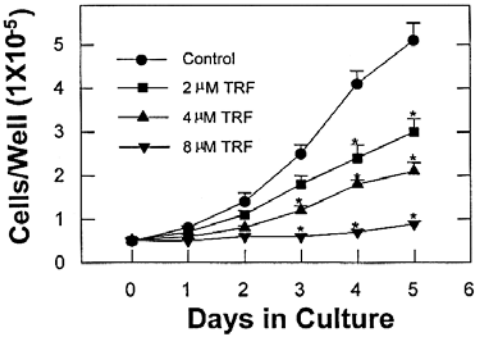


Figure 2. Effects of various doses of tocotrienol-rich-fraction of palm oil (TRF) on highly malignant +SA mammary epithelial cell proliferation in culture. Data points indicate the mean cell count/well ± SEM for 6 replicates in each treatment group throughout a 5 day culture period. *p < 0.05, as compared with controls.

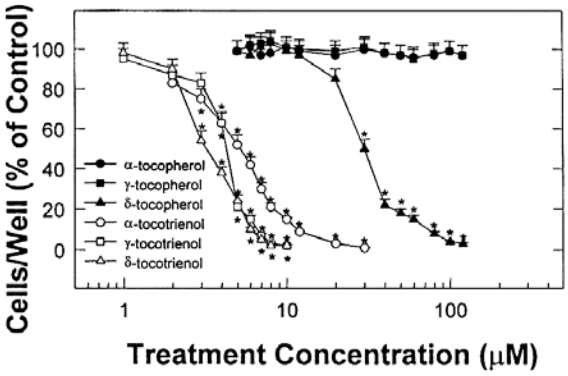


Figure 3. Effects of various doses of individual tocopherol and tocotrienol isoforms on highly malignant +SA mammary epithelial cell proliferation in culture. Data points indicate the percentage of viable cells/well ± SEM for 6 replicates in each treatment group, as compared with controls. *p<0.05, as compared with controls.

The effects of acute 24h exposure to various concentrations of tocopherols, tocotrienols, or TRF on viable +SA cell number are shown in Figure 4. Treatment for 24 h with 6-120 μM α-or γ-tocopherol had no affect, while treatment with 100-250 μM δ-tocopherol, 20-60 μM TRF, 20-60 μM α-tocotrienol, 15-60 μM γ-tocotrienol, or 8-60 μM δ-tocotrienol significantly decreased +SA viable cell number in a dose-responsive manner, as compared to controls (Figure 4).

These results demonstrate that individual tocopherol and tocotrienol isoforms display differential potencies in their antiproliferative and cytotoxic effects on the highly malignant +SA mammary epithelial cells grown in culture. Direct comparisons between the two vitamin E subclasses show that tocotrienols are significantly more potent than tocopherols. Furthermore, the biopotency of specific isoforms displayed a consistent relationship corresponding to δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol >> δ-tocopherol ≥ γ- and α-tocopherol.

Additional studies were conducted to determine whether tocotrienol-induced cytotoxicity in +SA cells resulted from the induction of apoptosis, as indicated by DNA fragmentation. DNA isolated from neoplastic +SA mammary epithelial cells exposed for 24 h to either control or 200μM α-tocopherol treatment media did not show appreciable levels of fragmentation, whereas treatment with cytotoxic doses of TRF (50 μM) or γ-tocotrienol (20 μM) induced intense DNA fragmentation in these cells.

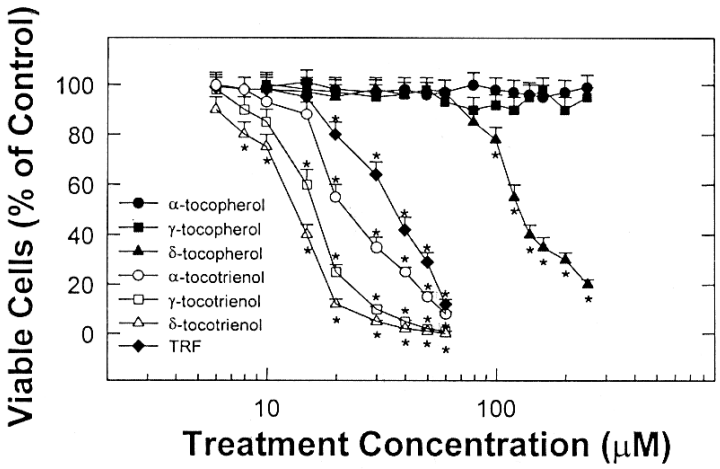


Figure 4. Highly malignant +SA mammary epithelial cell viability after a 24 h exposure period to various doses of tocotrienol-rich-fraction of palm oil (TRF), or individual tocopherol and tocotrienol isoforms. Cells in each treatment group were grown in culture and maintained on control medium for 5 day prior to exposure to their respective treatments. Data points indicate the percentage of viable cells/well ± SEM for six replicates in each treatment group, as compared with controls. *p<0.05, as compared to controls.

Treatment-induced apoptosis was verified in individual neoplastic +SA mammary epithelial cells using the TUNEL assay. Treatment with control or 200 μM α-tocopherol media resulted in the majority of +SA cell nuclei displaying light counterstaining with hematoxylin and only rare instances of dark positive TUNEL immunocytochemical staining (<5% of the cells). In contrast, acute 24 h treatment with 50 μM TRF or 20 μM γ-tocotrienol resulted in a large increase in

positive TUNEL immunocytochemical staining. Cell counts determined that TRF and γ-tocotrienol treatment resulted in 60% and 58% positive TUNEL immunocytochemical staining, respectively. Taken together, these results clearly demonstrate that tocotrienol-induced +SA cell death results from the induction of apoptosis.

It is well established that apoptosis can be initiated by different mechanisms in different cell types. Therefore, studies were conducted to determine whether caspase-8 (receptor-mediated) and/or caspase-9 (mitochondrial-stress) signaling pathways are involved in mediating tocotrienol-induced apoptosis. Neoplastic +SA mammary epithelial cells were exposed for 0-24 h with control, 50 μM TRF, 20 μM γ-tocotrienol, or 200 μM α-tocopherol treatment medium. TRF and γ-tocotrienol, but not α-tocopherol induced a large increase in caspase-8 and -3 activity within 2 h and continuing for at least 24 h after treatment exposure, as compared to the untreated controls. In contrast, these treatments had no affect on caspase-9 activity at any time during the 24 h treatment period. Western blot analysis showed that similar treatments with TRF or γ-tocotrienol induced large increases, whereas α-tocopherol had no affect on the relative levels of bioactive caspase-8 and -3 in +SA cells, as compared to untreated controls. Similar treatments had no affect on the relative levels of either inactive (49 kDa) or active (37 kDa) forms of caspase-9 in these cells.

Additional studies examined the effects of an acute 24 h treatment with either control 20 μM TRF, 200 μM α-tocopherol, or 20 μM γ-tocotrienol treatment medium alone or in combination with specific caspase inhibitors on +SA mammary epithelial cell viability. Acute treatment with either TRF or γ-tocotrienol significantly decreased viable +SA mammary epithelial cell number as compared to controls, and combined treatment with a specific caspase-8 inhibitor (1μM z-IETD-fmk) blocked this response. Similarly, TRF or γ-tocotrienol induced reductions in viable +SA cell number was blocked by combined treatment with a specific caspase-3 inhibitor (1μM z-DQMD-fmk). In contrast, combined treatment of TRF or γ-tocotrienol with a specific caspase-9 inhibitor (1μM z-LEHD-fmk) had no affect on tocotrienol-induced apoptosis in neoplastic +SA mammary epithelial cells.

Additional studies were conducted to determine the effects of selective caspase inhibitors on treatment-induced caspase levels and activation in neoplastic +SA mammary epithelial cells. Cells treated for 24 h with 50 μM TRF or 20 μM γ-tocotrienol alone displayed elevations in caspase-8 levels and activity, and combined treatment with a specific caspase-8 inhibitor, completely blocked this response. Similarly, treatment with TRY or γ-tocotrienol induced a relatively large increase in caspase-3 levels and activity, and this response was blocked by combined treatment with a specific caspase-3 inhibitor. In contrast, similar treatments, either alone or in combination with a specific caspase-9 inhibitor had no effect on caspase-9 activity in +SA cells.

These finding demonstrate that tocotrienol-induced apoptosis in neoplastic +SA mammary epithelial cells is initiated through receptor-mediated caspase-8 activation, and the subsequent down-stream activation of caspase-3. These results also show that mitochondrial stress-mediated activation of caspase-9 is not involved in mediating tocotrienol-induced programmed cell death in these cells.

DISCUSSION

These studies demonstrate that vitamin E derived from palm oil displays potent antiproliferative and apoptotic activity in highly malignant ±SA mammary epithelial cells. Furthermore, direct comparison between the two vitamin E subclasses showed that tocotrienols were significantly more potent in suppressing growth and inducing cell death than tocopherols. One possible explanation for the greater biopotency of tocotrienols versus tocopherols is suggested by the finding that tocotrienols are more easily or preferentially taken up by neoplastic mammary epithelial cells (McIntyre *et al.,* 2000a; McIntyre *et al.,* 2000b). Since tocotrienols differ from tocopherols in that they contain an unsaturated phytyl chain, the presence of these 3 double bonds might result in a less planar molecular conformation that facilitates less restricted transmembrane passage of tocotrienols into the cell, as compared to tocopherols. Since cellular accumulation of tocotrienols is greater than tocopherols, higher concentrations of tocotrienols are present at intracellular sites of action, thereby inducing a greater biological response. However, other studies showed that comparable intracellular levels of α-, γ-, or δ-tocopherol and α-, γ-, or δ-tocotrienol did not elicit similar antiproliferative and cytotoxic effects, suggesting that specific tocotrienol isoforms are inherently more potent than their corresponding tocopherol isoforms in reducing mitogenic-responsiveness and/or inducing apoptosis (McIntyre *et al.,* 2000a; McIntyre *et al.,* 2000b).

Neoplastic cells display greater sensitivity to the tocotrienol-induced apoptosis than normal mammary epithelial cells (McIntyre *et al.,* 2000a; McIntyre *et al.,* 2000b). Various signaling pathways have been suggested for mediating tocopherol- and tocotrienol-dependent apoptosis in different cell types (Chatelain *et al.,* 1993; Stauble *et al.,* 1994; Turley *et al.,* 1995; Fazzio, Marilley & Azzi, 1997; Sigounas, Anagnostou & Steiner 1997; Turley, Sanders & Kline 1997; Yu, Sanders & Kline 1997; Zhao *et al.,* 1997). The present study provides evidence that receptor- but not mitochondrial stress- mediated signaling pathways are involved in tocotrienol-induced apoptosis in highly malignant +SA mammary epithelial cells. Activation of death-receptors (e.g. Fas) stimulates the inactive form of caspase-8 in the cytoplasm to translocation to the cell surface receptor for activation (Earnshaw, Martins & Kaufman, 1999; Slee, Adrian & Martin 1999; Sun *et al.,* 1999; Bratton *et al.,* 2000). Active caspase-8 then activates effector caspases such as caspase-3, which subsequently mediate the various cytoplasmic and nuclear events associated with apoptosis. Alternatively, mitochondrial stress-induced caspase activation and apoptosis can be initiated by numerous cellular signals that cause perturbations in the mitochondria, release of cytochrome c into the cytoplasm, and ultimately the activation of caspase-9 (Earnshaw *et al.,* 1999; Slee *et al.,* 1999; Sun *et al.,* 1999; Bratton *et al.,* 2000). The present findings show that tocotrienols induced caspase-8 and -3, but not caspase-9 activation. Although, additional studies are required to determine the exact receptor(s) and ligand(s) involved in mediating tocotrienol-induced apoptosis, these findings suggest that palm oil derived tocotrienols may provide therapeutic value in reducing breast cancer risk and mortality in women.

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