

Tocotrienol-Induced Caspase-8 Activation Is Unrelated to Death Receptor Apoptotic Signaling in Neoplastic Mammary Epithelial Cells

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Tocotrienols, a subclass in the vitamin E family of compounds, have been shown to induce apoptosis by activating caspase-8 and caspase-3 in neoplastic mammary epithelial cells. Since caspase-8 activation is associated with death receptor apoptotic signaling, studies were conducted to determine the exact death receptor/ligand involved in tocotrienol-induced apoptosis. Highly malignant +SA mouse mammary epithelial cells were grown in culture and maintained in serum-free media. Treatment with 20 μ M γ -tocotrienol decreased +SA cell viability by inducing apoptosis, as determined by positive terminal dUTP nick end labeling (TUNEL) immunocytochemical staining. Western blot analysis showed that γ -tocotrienol treatment increased the levels of cleaved (active) caspase-8 and caspase-3. Combined treatment with caspase inhibitors completely blocked tocotrienol-induced apoptosis. Additional studies showed that treatment with 100 ng/ml tumor necrosis factor- α (TNF- α), 100 ng/ml FasL, 100 ng/ml TNF-related apoptosis-inducing ligand (TRAIL), or 1 μ g/ml apoptosis-inducing Fas antibody failed to induce death in +SA cells, indicating that this mammary tumor cell line is resistant to death receptor-induced apoptosis. Furthermore, treatment with 20 μ M γ -tocotrienol had no effect on total, membrane, or cytosolic levels of Fas, Fas ligand (FasL), or Fas-associated via death domain (FADD) and did not induce translocation of Fas, FasL, or FADD from the cytosolic to the membrane fraction, providing additional evidence that tocotrienol-induced caspase-8 activation is not associated with death receptor apoptotic signaling. Other studies showed that treatment with 20 μ M γ -tocotrienol induced a large decrease in the relative intracellular levels of phospho-phosphatidylinositol 3-kinase (PI3K)-dependent kinase 1 (phospho-PDK-1 active), phospho-Akt (active), and phospho-glycogen synthase kinase-

3, as well as decreasing intracellular levels of FLICE-inhibitory protein (FLIP), an antiapoptotic protein that inhibits caspase-8 activation, in these cells. Since stimulation of the PI3K/PDK/Akt mitogenic pathway is associated with increased FLIP expression, enhanced cellular proliferation, and survival, these results indicate that tocotrienol-induced caspase-8 activation and apoptosis in malignant +SA mammary epithelial cells is associated with a suppression in PI3K/PDK-1/Akt mitogenic signaling and subsequent reduction in intracellular FLIP levels. *Exp Biol Med* 229:745–755, 2004

Key words: tocotrienols; breast cancer; apoptosis; caspase-8; FLIP

Introduction

Previous investigations have demonstrated that tocotrienols, a subgroup in the vitamin E family of compounds, have potent apoptotic activity in neoplastic mammary epithelial cells (1–4). These findings are particularly interesting because they are observed using treatment doses that have little or no effect on normal mammary epithelial cell growth or viability (5, 6). Apoptosis is a highly regulated process executed by the activation of specific enzymes called caspases, a family of cysteine-dependent aspartyl proteases (7, 8). Caspases are constitutively present in cells as inactive proenzymes that must be cleaved and processed for activation (7, 8). Caspases can be classified as either initiator caspases (caspase-2, caspase-8, caspase-9, and caspase-10) that subsequently activate effector caspases (caspase-3, caspase-6, and caspase-7), which are responsible for most of the proteolytic cleavages associated with cellular self-destruction (7, 8).

Activation of initiator caspases can occur through two general mechanisms. The first mechanism involves death receptor activation and apoptotic signaling. Death receptors represent a subgroup in the tumor necrosis factor (TNF) superfamily of receptors that includes TNF-R1, Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptors, TRAIL-R1 and TRAIL-R2 (9, 10). Specific ligands to

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these death receptors include TNF- α that activates TNF-R1, Fas ligand (FasL) that activates Fas, and TRAIL that activates TRAIL-R1 and TRAIL-R2 (9, 10). Following activation, death receptors complex with adapter proteins such as Fas-associated via death domain (FADD) and initiator procaspases such as procaspase-8 to form the death-inducing signaling complex (DISC) at the membrane (9, 10). Within the DISC, procaspase-8 is cleaved to form the active p20 subunit of caspase-8 that subsequently cleaves and activates effector caspases, such as caspase-3, caspase-6, or caspase-7 (9, 10). The second mechanism mediating caspase activation is the "mitochondrial stress" apoptotic signaling pathway. Various stresses such as chemicals and radiation destabilize the outer mitochondrial membrane and cause the release of apoptotic proteins such as cytochrome-*c* and apoptosis-inducing factor (AIF) into the cytoplasm (11). Cytochrome-*c* then interacts with apoptotic protease activation factor-1 (APAF-1) and initiator procaspases, such as procaspase-9, to form the apoptosome. Following apoptosome formation, procaspase-9 is cleaved to form active caspase-9 subunit that is then released into the cytoplasm and activates downstream effector caspases (12).

Recent studies have shown that tocotrienol-induced apoptosis in neoplastic +SA mammary epithelial cells is mediated by specific intracellular signaling mechanisms that lead to sequential increase in activated caspase-8 and caspase-3 levels and activity (2, 4, 13). These studies also showed that tocotrienol-induced programmed cell death was not associated with an increase in activated caspase-9 levels or activity (2, 4, 13). Since caspase-8 processing and activation is associated with death receptor, whereas caspase-9 activation is associated with mitochondrial stress apoptotic signaling, these findings strongly suggest that tocotrienol-induced apoptosis is mediated by receptor-dependent caspase activation. However, the specific death receptor(s) and ligand(s) that mediate tocotrienol-induced caspase activation and apoptosis have not yet been determined (2, 4, 13).

Recently, it has been established that some cancer cells are resistant to death receptor-induced apoptosis (14–19). Death receptor resistance can result from a reduction in death receptors and/or ligand expression, death receptor gene mutation, or increased FLICE-inhibitory protein (FLIP) expression (14–19). FLICE-inhibitory protein is an endogenous inhibitor of caspase-8 activation that prevents the recruitment of procaspase-8 to the DISC and inhibits the processing of procaspase-8 to the active p20 subunit of caspase-8 (18–23). Studies have shown that activation of various growth factors and cytokine receptors stimulates the phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase 1 (PDK-1)/Akt mitogenic pathway, increases FLIP expression, promotes cell proliferation, and enhances cell survival (24–28). Phosphatidylinositol 3-kinase activates PDK-1 by phosphorylating Ser241 (29, 30). PDK-1 activates Akt by phosphorylating Thr308 and Ser473 (24,

29, 30). Although the exact mechanism by which Akt regulates FLIP expression is unknown, suppression of PI3K/PDK-1/Akt mitogenic signaling leads to a decrease in FLIP levels and a corresponding increase in caspase-8 activity (24, 25). Therefore, studies were conducted to determine the specific death receptor(s) and ligand(s) that are involved in mediating tocotrienol-induced caspase-8 activation and apoptosis in neoplastic +SA mammary epithelial cells. Additional studies examined the effects of tocotrienols on PI3K/PDK-1/Akt mitogenic signaling and FLIP expression in these cells.

Materials and Methods

Cell Line and Culture Conditions. The highly malignant +SA mammary epithelial cell line was derived from an adenocarcinoma that developed spontaneously in a BALB/c female mouse (31–33). Cell culture and experimental procedures have been previously described in detail (1, 3–6, 34, 35). Briefly, +SA cells were maintained in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 control media containing 5 mg/ml bovine serum albumin (BSA), 10 ng/ml epidermal growth factor (EGF), and 10 μ g/ml insulin. For subculturing, cells were rinsed twice with sterile Ca²⁺-free and Mg²⁺-free phosphate buffered saline (PBS) and incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 mins at 37°C. The isolated cells were washed once and then resuspended in serum-free media, and cells were counted by hemocytometer. Cells were plated at a density of 1×10^5 cells/well in 24-well culture plates (viability studies) or 1×10^6 cells/100-mm culture plates (Western blot and Akt kinase activity studies).

Chemicals. All chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) unless otherwise stated. Purified γ -tocotrienol was generously provided by Dr. Abdul Gapor of the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia). Caspase-8 inhibitor II (z-IETD-fmk), caspase-3 inhibitor V (z-DQMD-fmk), and LY294002 were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA); nonspecific caspase inhibitor (z-Asp-CH₂-DCB) and geldanamycin were purchased from Biomol Research Laboratories (Plymouth Meeting, PA); recombinant mouse TNF- α and Fas activation antibody were purchased from Chemicon International (Temecula, CA); recombinant human FasL plus and recombinant human TRAIL were purchased from Oncogene Research Products (San Diego, CA); antibodies for caspase-8, Fas, and FasL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); caspase-3 antibody was purchased from Trevigen (Gaithersburg, MD); antibodies for FADD and FLIP were purchased from Upstate Biotechnology (Lake Placid, NY); antibodies for phospho-PDK-1 (Ser241), total phosphatase and tensin homologue deleted from chromosome 10 (PTEN), phospho-PTEN (Ser380/Thr382/383), total Akt, and phospho-Akt (Ser473)

were purchased from Cell Signaling Technology (Beverly, MA); and anticytochrome oxidase subunit IV (COX) was purchased from MolecularProbes (Eugene, OR).

Experimental Treatments. In order to dissolve γ -tocotrienol in aqueous culture media, a stock solution of γ -tocotrienol was first prepared in sterile 10% BSA solution as previously described (1, 3–6, 34, 35). This solution of γ -tocotrienol/BSA was then used to prepare 0–20 μ M γ -tocotrienol-supplemented treatment media. Sterile stock solutions for the caspase inhibitors, LY294002, and geldanamycin were prepared in dimethyl sulfoxide (DMSO) and added to the culture media to obtain the final concentration. Stock solutions for TNF- α , Fas activation antibody, FasL, and TRAIL were prepared in sterile water and then added to the culture media to achieve the final concentration.

Measurement of Viable Cell Number. +SA cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (1, 3–6, 34, 35). Briefly, at the end of treatment exposure, cells were incubated at 37°C for 4 hrs with fresh control media containing 0.83 mg/ml MTT. After this incubation, the media was removed, MTT crystals were dissolved in 1 ml isopropanol, and the optical density of each sample was read at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company, Meriden, CT).

Terminal dUTP Nick End Labeling (TUNEL) Assay. Apoptosis was determined using the *in situ* cell death detection kit, POD (Roche Diagnostics Corporation, Indianapolis, IN) as previously described (4). Briefly, +SA cells were grown in serum-free control media to 60%–70% confluency on Lab-Tek chamber slides (Nalgene Nunc International, Naperville, IL), treated for 24 hrs with 0–20 μ M γ -tocotrienol, washed once with PBS, and then fixed with 4% paraformaldehyde. Slides were rinsed with PBS and air dried. TUNEL assay was performed, and slides were counterstained with hematoxylin. Photomicrographs were taken at 100 \times magnification. Positive TUNEL staining was quantified by image analysis using the Metamorph Analysis Software (Universal Imaging Corp., Downingtown, PA). The percentage of positive TUNEL cells was determined by counting the TUNEL positive cells versus the total number of cells in five photomicrographs (80,000 μ m² area per photomicrograph) for each treatment group.

Cell Lysis and Fractionation. +SA cells in the various treatment groups were isolated with trypsin and washed once with PBS as described above. Whole cell lysates were resuspended in lysis buffer, boiled for 5 mins, sonicated, and microcentrifuged at 4°C and supernatant was collected (1, 3–6, 34, 35). Subcellular fractionation was performed by resuspending cells in fractionation buffer and cells were disrupted by 30 strokes of Dounce homogenizer in ice as described previously (36). The lysates were then incubated on ice for 5 mins and centrifuged at 14,000 *g* for 1

hr at 4°C. Supernatants were collected as the cytosolic fractions. The remaining membrane fraction pellets were then dissolved in lysis buffer, boiled for 5 mins, sonicated, and microcentrifuged at 4°C, and supernatants were collected.

Western Blotting. +SA cells in the various treatment groups were isolated by trypsin or whole cell lysates or cytosolic and membrane fractions were prepared, and protein concentration was determined using a BioRad protein assay kit (BioRad, Hercules, CA). Samples (20 μ g for whole lysates and 10 μ g for membrane/cytosolic fractions) were loaded on 10%–15% polyacrylamide minigels and electrophoresed; Western blot analysis was performed as previously described (3, 4, 6, 34, 35); and scanning densitometric analysis was performed with Metamorph Analysis software (Universal Imaging Corp.).

Akt Kinase Activity Assay. Akt kinase activity in +SA cells was measured using an assay kit purchased from Cell Signaling Technology. Briefly, +SA cells were treated with 20 μ M γ -tocotrienol for 0–8 hrs. At the end of treatment exposure, cells were rinsed once with PBS and lysed on ice for 10 mins with the cell lysis buffer provided in the kit, and protein concentration was determined as described above. Akt was immunoprecipitated with immobilized Akt antibody from 200 μ g of protein per sample, pelleted by centrifugation, and then incubated for 30 mins at 30°C in 40 μ l kinase buffer supplemented with 200 μ M ATP and 1 μ g glycogen synthase kinase-3 (GSK-3) fusion protein. The reaction was terminated with 20 μ l sodium dodecyl sulfate (SDS) sample buffer, and the supernatant was isolated by microcentrifugation and then boiled for 5 mins. Samples (20 μ l) were electrophoresed on 12% polyacrylamide minigels, and phospho-GSK-3 α/β (Ser21/9) band was visualized by Western blot analysis.

Statistical Analysis. Differences among the various treatment groups in cell viability studies were determined by analysis of variance (ANOVA) followed by Dunnett's *t* test. Differences were considered statistically significant at a value of *P* < 0.05.

Results

Cell Viability and Apoptosis. The effects of 24-hr exposure to various concentrations of γ -tocotrienol on +SA cell viability are shown in Figure 1 (top). Treatment with 0–20 μ M γ -tocotrienol resulted in a dose-dependent decrease in +SA cell viability, with 20 μ M γ -tocotrienol inducing approximately 80% cell death (Figure 1, top). The effects of similar treatments on positive TUNEL immunocytochemical staining in +SA cells are shown in Figure 1 (bottom). Positive TUNEL staining in +SA cells was characterized by dark brown nuclear staining, in contrast to the light blue hematoxylin counterstaining observed in nonapoptotic cells (photomicrographs not shown). The number of +SA cells displaying positive TUNEL staining versus the total number of cells present in each sampling was determined for each

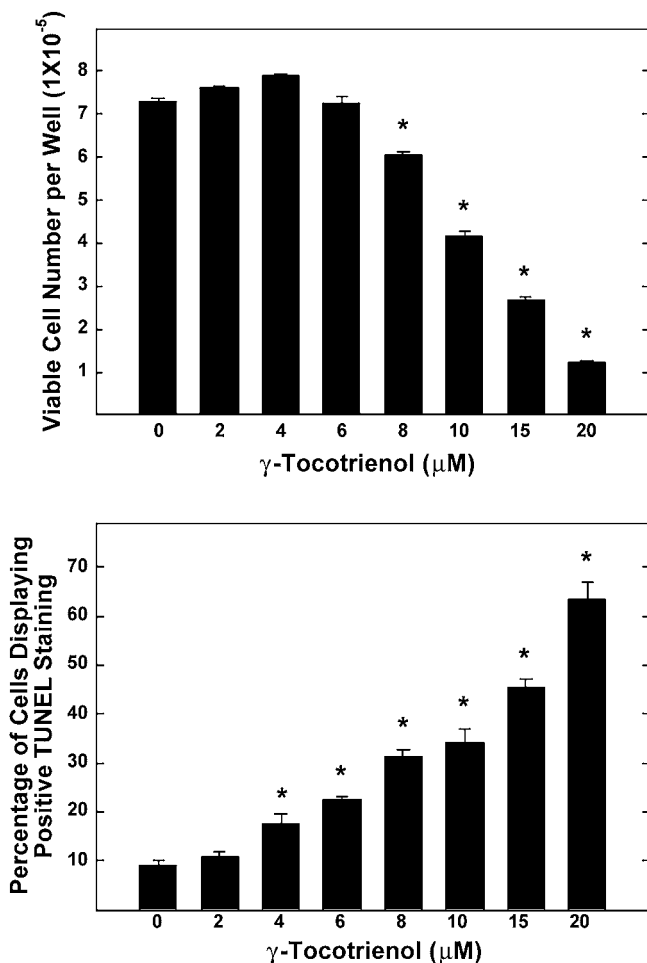


Figure 1. Effects of 24-hr exposure to 0–20 μM γ -tocotrienol on +SA cell viability are shown in the top panel. Cells were plated at a density of 1×10^5 cells/well (6 wells/group) and maintained on serum-free control media for 3 days. Afterward, cells were exposed to treatment media, and 24 hrs later viable cell number was determined using the MTT assay. The effects of similar treatments on the percentage of +SA cells displaying positive TUNEL staining are shown in the bottom panel. Cells were fixed with 4% paraformaldehyde prior to TUNEL assay. Cells were counted in five photomicrographs (80,000 μm^2 area per photomicrograph) for each treatment group. Vertical bars indicate the mean \pm SEM. * $P < 0.05$, as compared with controls.

treatment group (five samples/group). Treatment with 4–20 μM γ -tocotrienol induced a dose-responsive increase in positive TUNEL staining (Figure 1, bottom). Treatment with 20 μM γ -tocotrienol resulted in approximately 65% of the +SA cells displaying positive TUNEL staining, indicating that the majority of cells in this treatment group are actively undergoing apoptosis (Figure 1, bottom).

Caspase Activation. The effects of 20 μM γ -tocotrienol on intracellular levels of caspase-8, caspase-3, and FLIP during a 24-hr treatment period in +SA cells are shown in Figure 2. Western blot and scanning densitometric analyses showed that γ -tocotrienol treatment induced an increase in the relative levels of cleaved (active) p20 subunit of caspase-8 (Figure 2) and cleaved (active) p18 subunit of

caspase-3 (Figure 2) throughout the 24-hr treatment period. In contrast, γ -tocotrienol treatment decreased the relative levels of FLIP, an antiapoptotic protein that inhibits caspase-8 activation, during this same time period (Figure 2).

Caspase Inhibition and Cell Viability. Figure 3 shows the effects of combined treatment of γ -tocotrienol with caspase inhibitors on +SA cell viability following a 24-hr treatment exposure. Treatment with 20 μM γ -tocotrienol alone decreased +SA cell viability by approximately 80% as compared with untreated controls (Figure 3). Combined treatment with γ -tocotrienol and 5 μM of z-IETD-fmk (specific caspase-8 inhibitor, I-8), z-DQMD-fmk (specific caspase-3 inhibitor, I-3), or z-Asp-CH₂-DCB (nonspecific caspase inhibitor, I-All) blocked tocotrienol-induced apoptosis and corresponding decrease in viable +SA cell number (Figure 3).

Death Ligands, PI3K/Akt Inhibitors, and Cell Viability. Since caspase-8 activation is associated with death receptor (TNF-R1, Fas, TRAIL-R1, and TRAIL-R2) activation, studies were conducted to determine the susceptibility of +SA cells to death receptor ligands. Furthermore, since suppression of PI3K/PDK/Akt signaling is associated with decreased cellular survival and the induction of apoptosis, additional studies were also conducted to determine the effects of the PI3K inhibitor, LY294002, and indirect Akt inhibitor, geldanamycin, on +SA cell viability. Treatment for 24 hrs with a high dose (100 ng/ml) of death receptor ligand, TNF- α , FasL, or TRAIL had no effect on +SA cell viability as compared with untreated controls (Figure 4). Similarly, treatment for 24 hrs with 1 $\mu\text{g/ml}$ of Fas activating antibody also had no effect on +SA cell viability (Figure 4). In contrast, +SA cell viability was significantly decreased following a 24-hr exposure to 20 μM γ -tocotrienol, 20 μM LY294002, or 1 μM geldanamycin (Figure 4).

Death Receptor Apoptotic Signaling. The effects of 20 μM γ -tocotrienol on total, membrane, or cytosolic levels of proteins associated with Fas receptor signaling in +SA cells over an 8-hr treatment period are shown in Figure 5. The 0–8-hr treatment period was selected based on the previous finding that maximal caspase-8 processing and activation was observed within 8 hrs after γ -tocotrienol treatment exposure (4). Treatment with 20 μM γ -tocotrienol had no effect on the relative levels of Fas, FasL, or FADD in whole cell lysates (total) or cytosolic and membrane fractions (Figure 5). The FADD protein band was undetectable in the membrane fractions of +SA cells. Fas activation is associated with the translocation of FasL and FADD from the cytosolic to the membrane fraction of the cell. Fas activation can also be restored by enhanced Fas translocation from the cytosolic to the membrane fraction. However, γ -tocotrienol treatment was not found to induce Fas, FasL, or FADD translocation at any time during the 8-hr treatment period (Figure 5). In contrast, treatment with 20 μM γ -tocotrienol decreased the relative levels of FLIP in whole cell lysates and cytosolic fractions throughout the 8-

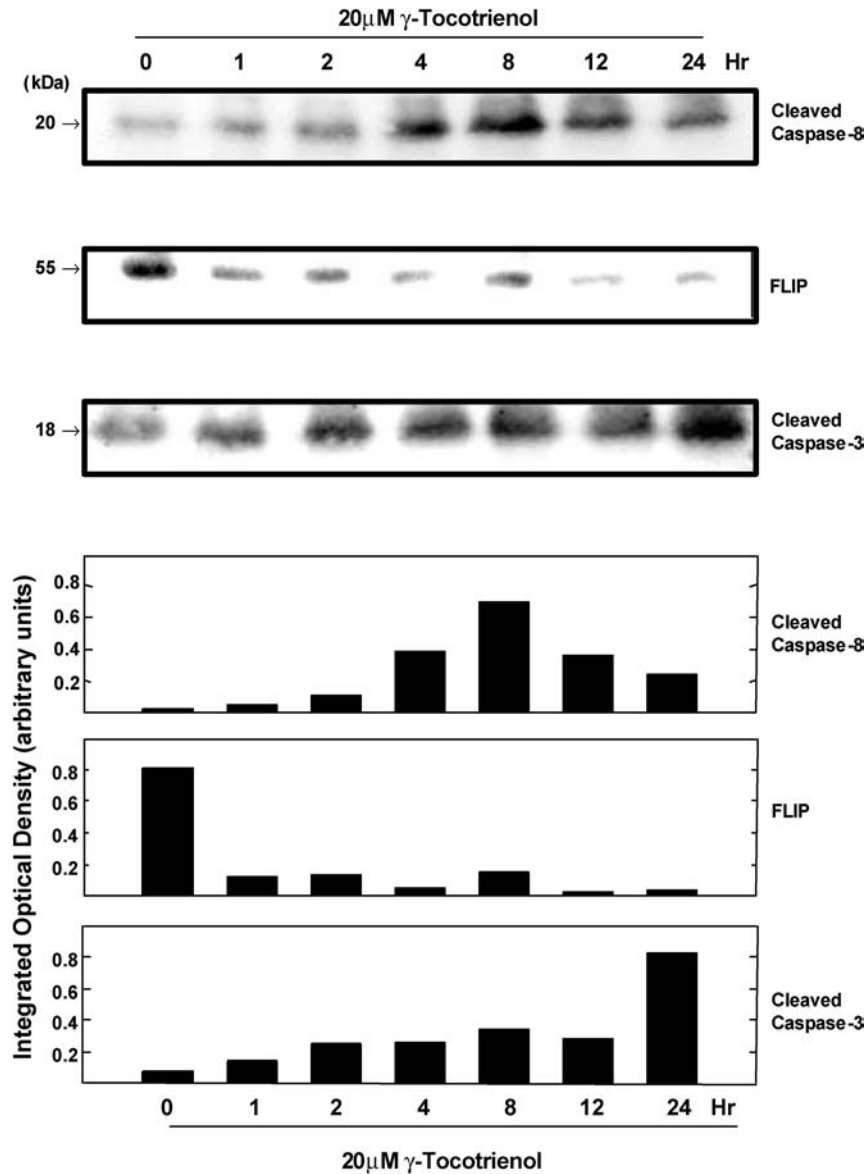


Figure 2. Western blot and scanning densitometric analysis of the relative levels of active, cleaved caspase-8; FLIP; and active, cleaved caspase-3 in +SA cells treated with 20 μM γ-tocotrienol for 0–24 hrs. Cells were grown in culture for 3 days in serum-free control media prior to treatment exposure. At various time points, whole cell lysates were prepared for subsequent fractionation by SDS–polyacrylamide gel electrophoresis (PAGE) (20 μg/well), followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and shown as bar graphs below the Western blots. Vertical bars in the densitometric graphs indicate the integrated optical density of bands visualized in each lane.

hr treatment period while reducing FLIP in the membrane fraction between 2 and 4 hrs after treatment exposure (Figure 5). Total FLIP levels decrease within 1 hr of treatment with γ-tocotrienol, whereas the FLIP levels in the cytosolic and membrane fractions start decreasing only 2 hrs after treatment (Figure 5). Although the reason for this reproducible finding is unknown, the fact remains that γ-tocotrienol treatment causes a large relative reduction in total, cytosolic, and membrane levels of FLIP in malignant +SA mammary epithelial cells. Western blot analysis of cytochrome *c* oxidase subunit IV (COX), an enzyme located on the mitochondrial membrane, was used as a marker for

subcellular fractionation purity. The relative levels of COX in the membrane fraction were high prior to and after γ-tocotrienol treatment exposure, while cytosolic levels of COX were very low throughout the experiment (Figure 5). These results demonstrate that subcellular fractionation of +SA cells was relatively pure and that the cytosolic fraction was free of membrane fraction contamination.

PI3K/PDK-1/Akt Mitogenic Signaling. Since stimulation of the PI3K/PDK-1/Akt mitogenic signaling pathway is associated with increased FLIP expression, it was of interest to determine the effects of γ-tocotrienol on PI3K/PDK/Akt signaling in +SA cells. Additional studies were

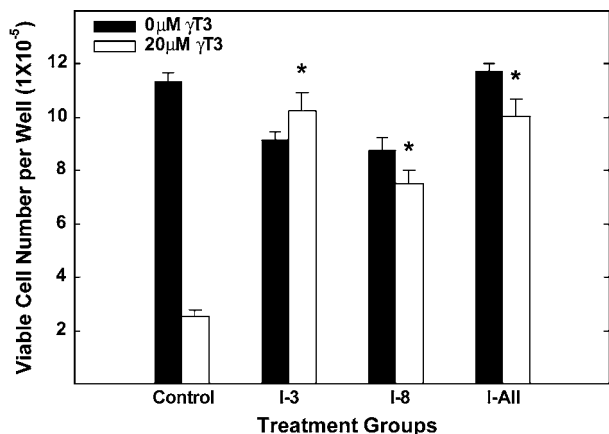


Figure 3. Effects of 24 hrs exposure to 0 μM (closed bars) or 20 μM (open bars) γ -tocotrienol alone or in combination with caspase inhibitors on +SA cell viability. Cells were plated at a density of 1×10^5 cells/well and maintained in culture for 3 days on serum-free control media. Afterward, media were replaced with fresh treatment media containing either 0 or 20 μM γ -tocotrienol and vehicle (control); 5 μM z-DQMD-fmk, a specific caspase-3 inhibitor (I-3); 5 μM z-IETD-fmk, a specific caspase-8 inhibitor (I-8); or 5 μM z-Asp-CH₂-DCB, a nonspecific caspase inhibitor (I-All), and 24 hrs later cell viability was determined using the MTT assay. Vertical bars indicate mean \pm SEM for six replicates in each treatment group. * $P < 0.05$, as compared with 20 μM γ -tocotrienol treated controls.

also conducted to determine the effects of γ -tocotrienol on PTEN phosphatase activity, an enzyme known to suppress PI3K activity. Western blot analysis showed that treatment with 20 μM γ -tocotrienol resulted in a large time-dependent decrease in the relative levels of phospho-PDK-1 (active) during the 8-hr treatment period (Figure 6). However, γ -tocotrienol treatment had no effect on total or phospho-PTEN levels in +SA cells (Figure 6). This same treatment only marginally decreased the total (active and inactive) levels of Akt but did cause a large decrease in the relative levels of phospho-Akt (active) starting at 4 hrs and continuing for at least 8 hrs following γ -tocotrienol exposure (Figure 6). Treatment effects on Akt activity, as determined by phospho-GSK-3 (Ser 9 and Ser 21) protein levels, are shown in Figure 6. Western blot analysis showed that treatment with 20 μM γ -tocotrienol induced a large decrease in the relative levels of phosphorylated GSK-3 protein after 4–8 hrs of treatment exposure, indicating a reduction in Akt activity (Figure 6).

Discussion

Results in this study demonstrate that γ -tocotrienol-induced apoptosis in malignant +SA mouse mammary epithelial cells is mediated by caspase-8 and caspase-3 activation. Since processing and activation of caspase-8 is generally associated with death receptor apoptotic signaling, it was initially hypothesized that γ -tocotrienol stimulates death receptor (TNF-R1, Fas, TRAIL-R1, and/or TRAIL-R2) activation. However, treatment with high doses of FasL or Fas activating antibody did not induce apoptosis in +SA cells. Similarly, treatment with high doses of additional death

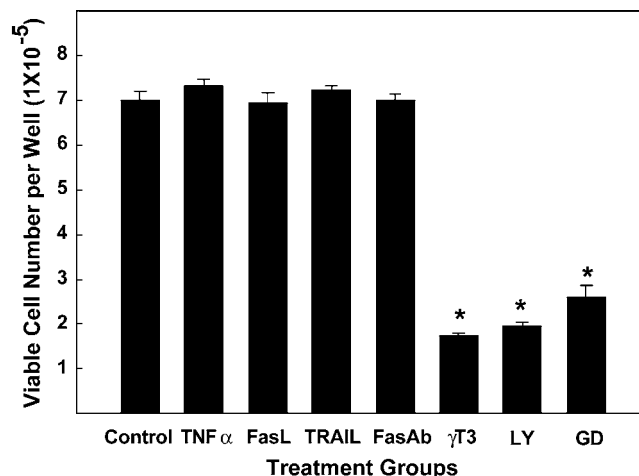


Figure 4. Comparative effects of 24-hr treatment exposure to γ -tocotrienol, various death receptor ligands, LY294002 (PI3K inhibitor), or geldanamycin (indirect Akt inhibitor) on +SA cell viability. Cells were plated at a density of 1×10^5 cells/well and maintained in culture for 3 days on serum-free control media. Afterward, media were replaced with fresh treatment media containing vehicle (control), 100 ng/ml TNF- α , 100 ng/ml FasL, 100 ng/ml TRAIL, 1 $\mu\text{g/ml}$ Fas activating antibody (FasAb), 20 μM γ -tocotrienol (γ T3), 20 μM LY294002 (LY), or 1 μM geldanamycin (GD), and 24 hrs later cell viability was determined using the MTT assay. Vertical bars indicate mean \pm SEM for six replicates in each treatment group. * $P < 0.05$, as compared with vehicle treated controls.

receptor ligands, such as TRAIL and TNF- α , was also found to have no adverse effect on +SA cell viability. Experimental findings also showed that treatment with apoptosis-inducing doses of γ -tocotrienol (20 μM) did not cause increased expression of Fas, FasL, or FADD and failed to induce translocation of Fas, FasL, or FADD from the cytosolic to the membrane fraction in +SA cells. These findings indicate that Fas, TRAIL-R1, TRAIL-R2, and TNF-R1 death receptors are nonfunctional in +SA cells, and γ -tocotrienol-induced caspase activation and apoptosis does not result from a restoration in apoptotic signaling by Fas. However, additional studies showed that treatment with 20 μM γ -tocotrienol decreased PI3K/PDK-1/Akt mitogenic signaling and FLIP expression in these cells. Taken together, these findings suggest that γ -tocotrienol-induced caspase-8 activation and apoptosis in +SA cells does not result from enhanced death receptor activation but is instead associated with tocotrienol-induced suppression of PI3K/PDK-1/Akt mitogenic signaling and the subsequent downregulation of FLIP, an endogenous inhibitor of caspase-8 processing and activation.

These results confirm and extend previous investigations demonstrating tocotrienol-induced apoptosis is mediated by caspase-8 and caspase-3 activation and is independent of caspase-9 activation (4, 13). Although previous investigations did not determine the specific death receptor(s) and ligand(s) involved in mediating tocotrienol-dependent caspase activation, it is well established that FasL activation of the death receptor, Fas, stimulates procaspase-8 activation to caspase-8 (9, 10). Previous studies have also shown that Fas activation plays a role in normal mammary

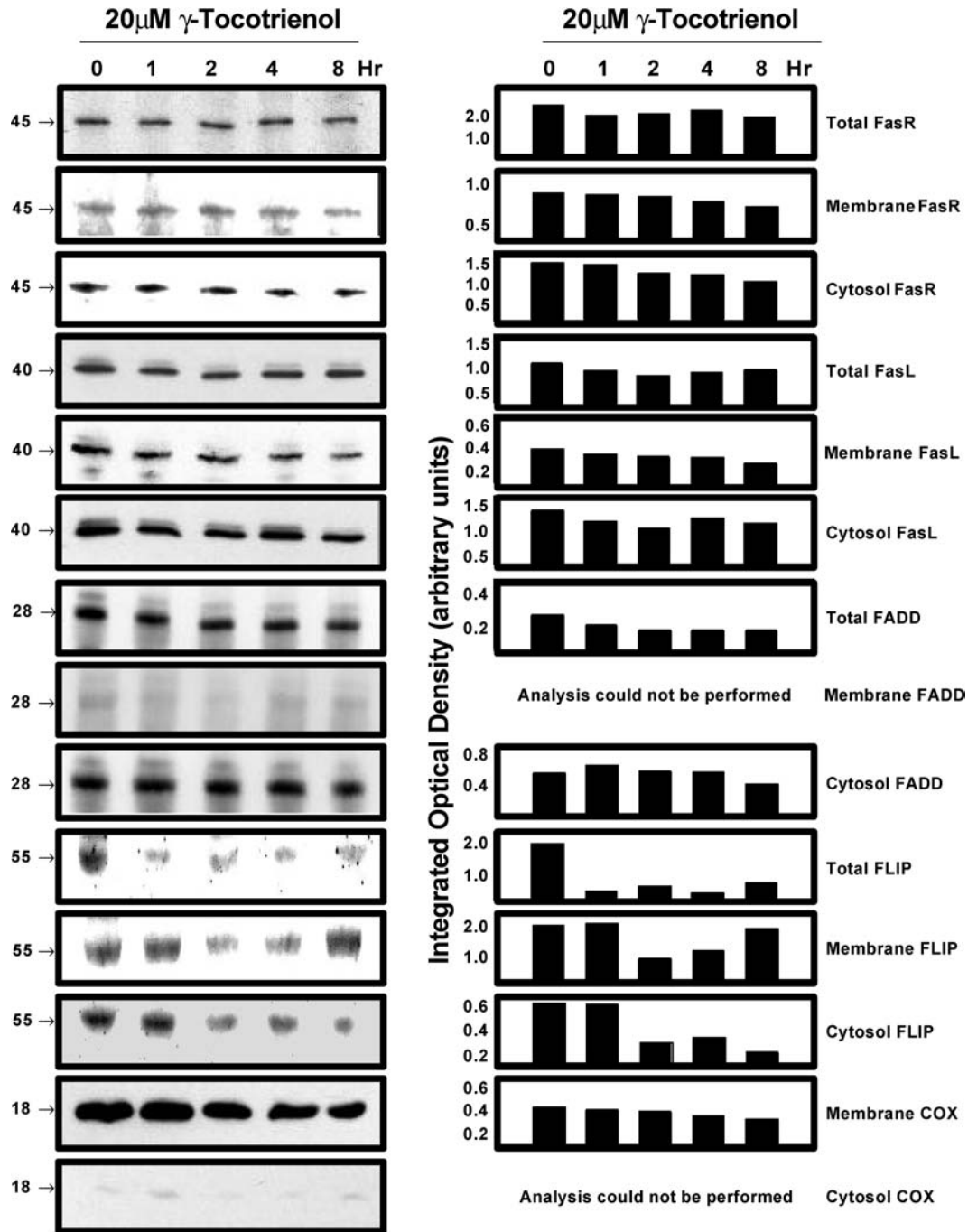


Figure 5. Western blot and scanning densitometric analysis of the relative total, membrane, or cytosolic levels of FasR, FasL, FADD, FLIP, and COX in +SA cells treated with 20 μM γ-tocotrienol for 0–8 hrs. Cells were grown in culture for 3 days in serum-free control media prior to treatment exposure. At various time points, whole cell lysates or membrane and cytosolic fractions were prepared for subsequent fractionation by SDS-PAGE (20 μg/well for whole cell lysates, and 10 μg/well for membrane/cytosolic fractions), followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and is presented as bar graphs adjacent to the corresponding Western blot. Vertical bars in the densitometric graphs indicate the integrated optical density of bands visualized in each lane.

gland remodeling and involution, as well as in the removal of neoplastic mammary epithelial cells during the early stages of tumorigenesis (37). In addition, treatment with the succinate derivative of RRR-α-tocopherol, RRR-α-tocopheryl succinate, has been shown to induce DNA synthesis arrest and apoptosis in breast cancer cells, and these effects

are associated with Fas apoptotic signaling (36, 38). The present study demonstrates for the first time that the death receptor, Fas, is nonfunctional in malignant +SA mammary epithelial cells and that tocotrienol-induced caspase-8 and caspase-3 activation occurs independently of Fas activating and apoptotic signaling. Experimental data showed that

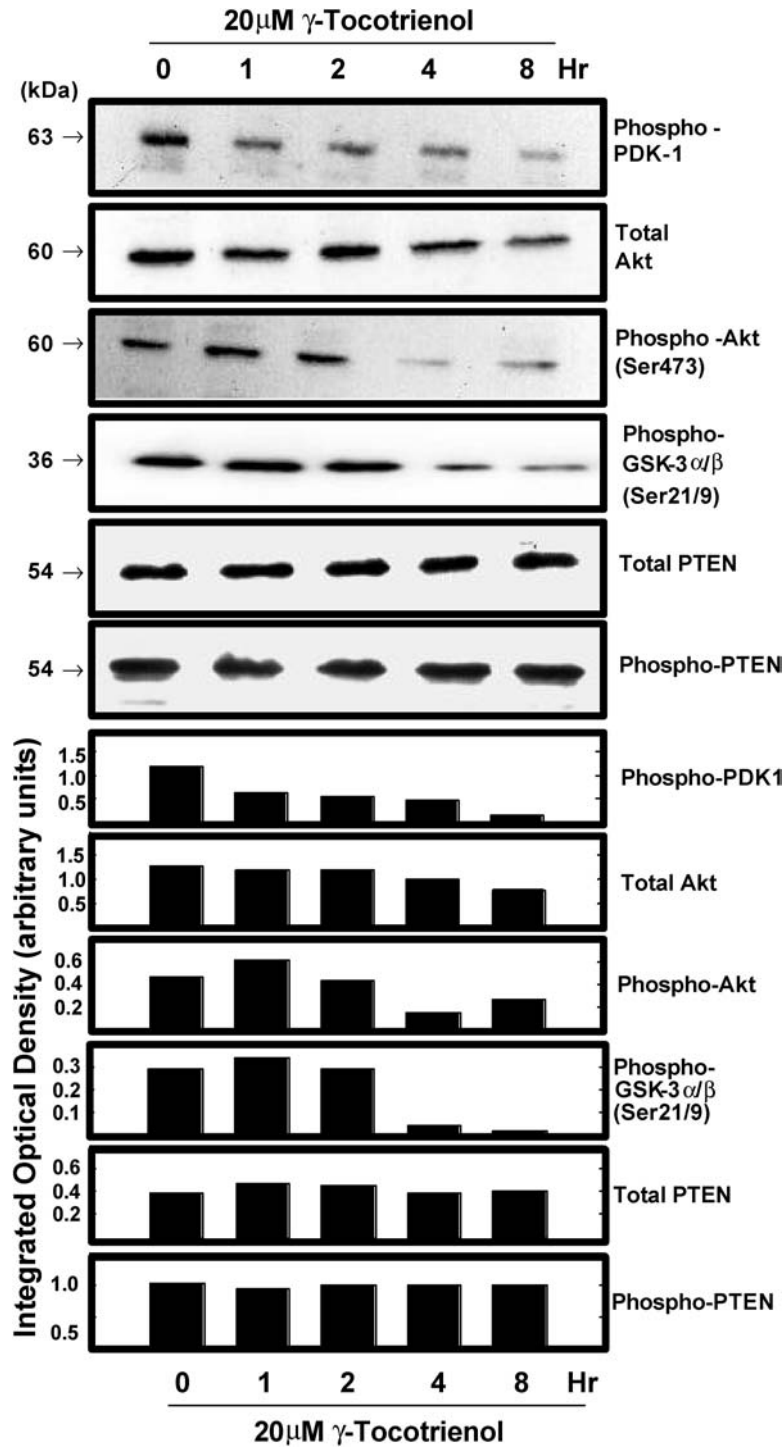


Figure 6. Western blot and scanning densitometric analysis showing the effects of 0–8 hrs of 20 μ M γ -tocotrienol treatment on total relative levels of phospho-PDK-1 (active form, Ser241), total PTEN, phospho-PTEN (inactive form, Ser380/Thr382/383), total Akt, phospho-Akt (active form, Ser473), and Akt kinase activity in +SA cells. For phospho-PDK-1, PTEN, phospho-PTEN, Akt, and phospho-Akt experiments, cells were grown in culture for 3 days in serum-free control media prior to treatment exposure. At various time points, whole cell lysates were prepared for subsequent fractionation by SDS-PAGE (20 μ g/well), followed by Western blot analysis. Cells were prepared in a similar manner for assay of Akt activity, except that Akt was immunoprecipitated from whole cell lysates and incubated with GSK-3 fusion protein and ATP prior to Western blot analysis for phospho-GSK-3- α/β (Ser21/9). Scanning densitometric analysis was performed for each blot and is presented as bar graphs below the Western blots. Vertical bars in the densitometric graphs indicate the integrated optical density of bands visualized in each lane.

stimulation of Fas with very high doses of Fas activation antibody or FasL had no effect on +SA cell viability. Likewise, treatment with 20 μ M γ -tocotrienol (cytotoxic dose) had no effect on intracellular Fas, FasL, and FADD levels. Previous studies have shown that RRR- α -tocopheryl succinate restores Fas sensitivity and apoptotic signaling in Fas-insensitive human breast cancer cells (36). However, the present study demonstrates that tocotrienol treatment does not restore Fas receptor signaling in Fas-insensitive malignant +SA mammary epithelial cells, as indicated by the absence of Fas, FasL, or FADD translocation from the cytosolic to membrane fractions of +SA cells.

It has also been shown that treatment with TRAIL induces caspase-8 activation by stimulating TRAIL-R1 and TRAIL-R2 apoptotic signaling in several cell types (9, 10). Likewise, TNF- α has been shown to stimulate TNF-R apoptotic signaling and caspase activation (9, 10). However, treatment with high doses of TNF- α or TRAIL was found to have no effect on +SA cell viability, suggesting that tocotrienol-induced caspase activation in these cells occurs independently of TNF-R and TRAIL-R apoptotic signaling. Although these data provide strong evidence, additional knockdown studies, such as those involving the use of death receptor neutralizing antibodies, antisense oligomers, or small interfering RNA (siRNA), are required to conclusively determine whether tocotrienol-induced caspase activation occurs independently of death receptor apoptotic signaling in malignant +SA mammary epithelial cells. Nevertheless, the results from this present study suggest that tocotrienol treatment might also be effective in inducing apoptosis and reducing viability in death receptor resistant human breast cancer.

Resistance to death receptor-induced apoptosis is associated with enhanced tumorigenesis, multidrug resistance, and enhanced survival in a number of tumor cell type cancers (14, 15, 36). A reduction in the expression of death receptors and/or their ligands, as well as mutations within genes encoding for death receptors, is associated with insensitive or nonfunctional death receptors in various types of tumor cells (14–17). In addition, resistance to apoptotic stimuli can be acquired through upregulation and enhanced activity of various mitogen-dependent signaling pathways, particularly the PI3K/PDK/Akt mitogenic signaling pathway (24–30, 39, 40). Stimulation of the PI3K/PDK/Akt pathway has been shown to increase expression of antiapoptotic protein, FLIP, which inhibits procaspase-8 cleavage and activation (24–30, 39, 40). Previous investigations have also shown that overexpression of FLIP is associated with tumor cell resistance to death receptor activation and apoptotic signaling and that the ratio between caspase-8 and FLIP levels is critical in determining tumor cell sensitivity to apoptotic stimuli (18–23). The present study shows that treatment with apoptosis-inducing doses of γ -tocotrienol increases the ratio of caspase-8 versus FLIP in malignant +SA mammary epithelial cells. Treatment with 20 μ M γ -tocotrienol resulted in a large and

prolonged decrease in intracellular FLIP levels and a corresponding increase in intracellular cleaved (active) caspase-8 levels within 24 hrs of treatment.

It is well established that various intracellular signaling pathways and second messengers associated with cellular proliferation and survival, such as Ras/Raf/MEK/ERK, PI3K/PDK-1/Akt, and NF κ B, modulate FLIP expression (24, 25, 41, 42). A common characteristic of many breast cancer cells is elevated PI3K/PDK-1/Akt signaling, and pharmacologic treatments that inhibit PI3K/PDK-1/Akt signaling have been shown to reduce intracellular FLIP expression, increase caspase-8 activation, and reduce cell survival (24–30, 39, 40). The present findings show that treatment with apoptosis-inducing doses of γ -tocotrienol caused a relatively large decrease in phospho-PDK-1 (active form), and phospho-Akt (active form), a reduction in Akt kinase activity, and a corresponding decrease in FLIP expression in +SA cells. However, γ -tocotrienol did not lead to an increase in the activity of PTEN, a lipid phosphatase known to inhibit PI3K activity, suggesting that tocotrienol-induced suppression in PI3K/PDK-1/Akt signaling does not result from an increase in PTEN activity. These data indicate that γ -tocotrienol-induced caspase-8 activation and apoptosis are associated with suppression in PI3K/PDK-1/Akt signaling and a decrease in FLIP expression in +SA cells. At present, it is not known whether γ -tocotrienol directly inhibits PDK-1 activity or acts at some upstream site to inhibit PDK-1 activation. However, it is known that PI3K activation is stimulated by various growth factors, particularly EGF (24, 26–28). Previous studies have shown that γ -tocotrienol treatment had no effect on EGF-receptor levels or EGF-receptor tyrosine kinase activity but did attenuate EGF-dependent mitogenic signaling in normal and preneoplastic mammary epithelial cells, (2, 3, 6, 13). Further studies are needed to determine the exact site of action where γ -tocotrienol acts to suppress EGF-dependent PI3K/PDK-1/Akt signaling in malignant +SA mammary epithelial cells.

In conclusion, these findings demonstrate that γ -tocotrienol-induced caspase-8 activation and apoptosis is not associated with death receptor signaling in malignant +SA mouse mammary epithelial cells. Experimental findings indicate that tocotrienol-induced caspase-8 activation results from the suppression of PI3K/PDK-1/Akt mitogenic signaling and a corresponding decrease in intracellular FLIP expression, an endogenous inhibitor of caspase-8 activation. Recent studies have shown that resistance to death receptor-induced apoptosis is an important contributor for multidrug resistance in a variety of cancer cell types, and nonfunctional death receptors are also associated with cancer cell escape and survival from normal immunosurveillance mechanisms (14, 15, 36). Therefore, it is possible that tocotrienols may provide additional therapeutic benefits when combined with traditional chemotherapeutic agents in the treatment of breast cancer in women. Understanding the molecular mechanisms involved in tocotrienol-induced apoptosis may also provide critical leads for the development of novel therapies to treat

breast cancer cases that have acquired resistance to death receptor signaling.

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