# γ-Tocotrienol Inhibits Neoplastic Mammary Epithelial Cell Proliferation by Decreasing Akt and Nuclear Factor κB Activity

SUMIT J. SHAH AND PAUL W. SYLVESTER[1]

*School of Pharmacy, University of Louisiana at Monroe, Monroe, Louisiana 71209–0470*

**Tocotrienols, a subgroup within the vitamin E family of compounds, have been shown to display potent anticancer activity and inhibit preneoplastic and neoplastic mammary epithelial cell proliferation at treatment doses that have little or no effect on normal cell growth and function. However, the specific intracellular mechanisms mediating the antiproliferative effects of tocotrienols are presently unknown. Because Akt and nuclear factor κB (NFκB) are intimately involved in mammary tumor cell proliferation and survival, studies were conducted to determine the effects of γ-tocotrienol on Akt and NFκB activity in neoplastic +SA mammary epithelial cells *in vitro*. Treatment with 0–8 μ*M* γ-tocotrienol for 0–3 days caused a dose-responsive inhibition in +SA cell growth and mitotic activity, as determined by MTT colorimetric assay and proliferating cell nuclear antigen immunocytochemical staining, respectively. Studies also showed that treatment with 4 μ*M* γ-tocotrienol, a dose that inhibited +SA cell growth by more than 50% compared with that of untreated control cells, decreased intracellular levels of activated phosphotidylinositol 3-kinase-dependent kinase (PI3K)-dependent kinase 1 (phospho-PDK-1) and Akt, and reduced phospho-Akt kinase activity. Furthermore, these effects were not found to be associated with an increase in either phosphatase and tensin homologue deleted from chromosome 10 (PTEN) or protein phosphatase type 2A phosphatase activity. In addition, γ-tocotrienol treatment was shown to decrease NFκB transcriptional activity, apparently by suppressing the activation of IκB-kinase-α/β, an enzyme associated with inducing NFκB activation. In summary, these findings demonstrate that the antiproliferative effects of γ-tocotrienol result, at least in part, from a reduction in Akt and NFκB activity in neoplastic +SA mammary epithelial cells.** Exp Biol Med 230:235–241, 2005

**Key words:** tocotrienols; vitamin E; breast cancer; Akt; NFκB

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[1]To whom correspondence should be addressed at School of Pharmacy, 700 University Avenue, University of Louisiana at Monroe, Monroe, LA 71209–0470. E-mail: sylvester@ulm.edu

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Previous investigations demonstrated that tocotrienols, a subgroup within the vitamin E family of compounds, inhibit the growth of preneoplastic and neoplastic mammary epithelial cells (1–4). These findings are of particular interest because these antiproliferative effects were observed using treatment doses that had little or no effect on normal mammary epithelial cell growth or viability (5, 6). The relative selectivity displayed by tocotrienols against mammary tumor cells suggests that these compounds may provide significant therapeutic benefit in the prevention or treatment (or both) of breast cancer in women. However, the specific intracellular mechanism or mechanisms mediating the antiproliferative effects of tocotrienols has not yet been determined.

Several signaling pathways have been identified that are involved in mediating mitogen-dependent growth and survival in neoplastic mammary epithelial cells, particularly the phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase (PDK)/Akt, and nuclear factor κB (NFκB) signaling pathways (7–11). Elevated PI3K/PDK/Akt or NFκB signaling (or both) have been correlated with advanced tumor progression and poor prognosis in patients with breast cancer (12–14). PI3K is a family of lipid signaling kinases that generate various membrane lipid products, such as phosphatidylinositol-3,4-diphosphate ($PIP_2$) and phosphatidylinositol-3,4,5-triphosphate ($PIP_3$) (15, 16). PDK-1 preferentially binds to $PIP_3$ in the cell membrane and is then rapidly phosphorylated and activated by PI3K (17). Activated PDK-1 then phosphorylates and activates Akt (17). In addition, Akt can bind to $PIP_2$, which enhances PDK-1 phosphorylation and activation of Akt (17). Activated Akt has been shown to phosphorylate various proteins associated with cell survival and proliferation of both normal and neoplastic mammary epithelial cells (13, 17–19). Akt inactivation is primarily regulated by two phosphatases, phosphatase and tensin homologue deleted from chromosome 10 (PTEN), and protein phosphatase type 2A (PP2A) (18). PTEN inhibits the activation of PDK-1, whereas PP2A can inhibit $PIP_2$-mediated phosphorylation of Akt (18). Signaling of NFκB is also a complex, multistep process (20). NFκB exists in an inactive state in most cells bound to its inhibitory protein, IκB (20). Activation of

NFκB can be initiated by several different kinases, such as Akt and NFκB-inducing kinase (NIK), which induce the formation and activation of the ultimate kinase complex, IκB-kinase (IKK), which is composed of IKK-α, IKK-β, and the regulatory IKK-γ (19–21). Activated IKK phosphorylates IκB and promotes its degradation, leading to the release and enhanced phosphorylation and activation of NFκB (19–21). Activated NFκB translocates to the nucleus and binds to the DNA to initiate expression of genes that enhances cell proliferation and survival (14, 20, 22).

Because epidermal growth factor (EGF)-dependent mitogenesis is associated with the activation of Akt and NFκB, it was hypothesized that tocotrienol inhibition of EGF-dependent neoplastic mammary epithelial cell proliferation may result from the suppression of Akt or NFκB mitogenic signaling (or both). Because specific phosphatases such as PTEN and PP2A are associated with the regulation of Akt activation, studies were conducted to determine the effects γ-tocotrienol on Akt and NFκB activity, as well as PTEN and PP2A phosphatase activity in highly malignant +SA mouse mammary epithelial cells.

## Materials and Methods

**Reagents and Antibodies.** All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Purified γ-tocotrienol was provided as a gift by Dr. Abdul Gapor at the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia). Antibodies for phospho-PDK-1 (Ser241), total Akt, phospho-Akt (Ser473), total PTEN, phospho-PTEN (Ser380/Thr382/383), phospho-IKK-α/β (Ser180/181), phospho-IκB-α (Ser32/36), and phospho-NFκB (Ser536) p65 subunit were purchased from Cell Signaling Technology (Beverly, MA), and anti-PP2Ac was purchased from Upstate Biotechnology (Lake Placid, NY).

**Cell Line and Culture Conditions.** Cell culture and experimental procedures have been previously described in detail (1, 3, 4, 23, 24). Serum-free defined control media consisting of Dulbecco modified Eagle medium/F12 containing 5 mg/ml bovine serum albumin, 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml epidermal growth factor (EGF), and 10 μg/ml insulin. Cells were plated at a density of $5 \times 10^4$ cells/well in 24-well culture plates (viability studies) or $1 \times 10^6$ cells/100-mm culture plates (all other studies) and allowed to attach in serum-free control media for 24 hrs. Cells were then divided into different treatment groups and fed control media or treatment media every other day, and maintained in a humidified incubator at 37°C in an environment of 95% air and 5% $CO_2$. The highly lipophilic γ-tocotrienol was dissolved in culture media as described previously (1–6). +SA cell number was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (1–6, 25, 26).The number of cells per well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometry, at the start of each experiment. Each experiment was repeated at least three times.

**Proliferating Cell Nuclear Antigen (PCNA) Assay.** Extent of cell proliferation or mitotic activity of +SA cells was determined using the PCNA staining kit (Zymed Laboratories, South San Francisco, CA). Briefly, nonconfluent cells were isolated by trypsinization, and $1 \times 10^6$ cells were plated and allowed to attach in serum-free defined control media for 24 hrs on Lab-Tek chamber slides (Nalgene Nunc International, Naperville, IL), then treated for 0–3 days with 4 μ*M* γ-tocotrienol, washed once with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde. Slides were rinsed with PBS and air-dried. PCNA assay was performed and slides were counterstained with hematoxylin. Photomicrographs were taken at ×200 magnification. Positive PCNA staining was quantified by image analysis using the Metamorph Analysis Software (Universal Imaging Corporation, Downingtown, PA). The percentage of proliferating cells was determined by counting the PCNA-positive cells versus the total number of cells in five photomicrographs (80,000 $\mu m^2$ area per photomicrograph) for each treatment group.

**Western Blot Analysis.** Neoplastic +SA cells in the various treatment groups were isolated by trypsinization, lysed with a whole cell lysis buffer (620.5 m*M* Tris-HCl pH 6.8, 2% w/v sodium dodecyl sulfate, 10% glycerol, 5% v/v 2-mercaptoethanol, and 2 m*M* sodium orthovanadate), boiled for 5 mins, sonicated, and microcentrifuged at 4°C. The supernatant was collected as the whole cell lysate, and protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples (20 μg/lane) were loaded on 7.5%–15% polyacrylamide minigels, electrophoresed, and Western blot and scanning densitometric analyses were performed as previously described (2–4).

**Akt Kinase Activity Assay.** Akt kinase activity in +SA cells was measured using a nonradioactive assay kit purchased from Cell Signaling Technology. Cells in culture were treated with 4 μ*M* γ-tocotrienol for 0–3 days. At the end of treatment exposure, cells were rinsed once with PBS, lysed on ice for 10 mins with the cell lysis buffer provided in the kit, and protein concentration was determined as described above. Immunoprecipitation of Akt and assay of Akt activity was previously described in detail (4).

**NFκB Transcriptional Activity Assay.** NFκB activity in +SA cells treated for 0–3 days with 4 μ*M* γ-tocotrienol was measured using a colorimetric TransAM NFκB p65 Transcription Factor Assay kit (Active Motif, Carlsbad, CA). Following treatment exposure, +SA cells in each treatment group were washed once, then scraped and collected in 10 ml of ice-cold PBS/phosphatase-inhibitor buffer (PIB) supplied in the kit. Cells were pelleted and then lysed on ice for 15 mins with 1 ml of ice-cold hypotonic buffer provided in the kit. Nonidet P-40 was added (0.5% v/v), and the crude lysate was microcentrifuged for 30 secs at 4°C. The supernatant was discarded; the nuclear pellet was

resuspended in complete lysis buffer, and incubated for 30 mins on a rocking platform at 4°C. The nuclear suspension was then microcentrifuged for 10 mins at 14,000 *g* at 4°C, and the supernatant was used as the nuclear extract. The protein concentration of nuclear extracts was determined as described above, and 10 μg of protein per sample in triplicate was used to measure NFκB activity in a 96-well plate, precoated with an oligonucleotide containing the NFκB p65 subunit consensus binding site (27). The active NFκB present in the +SA nuclear extracts binds to this consensus oligonucleotide, and NFκB activity was measured as optical density units read at 450 nm.

**Statistical Analysis.** Differences among the various treatment groups were determined by analysis of variance followed by the Dunnett *t* test. Differences were considered statistically significant at a value of $P < 0.05$.

## Results

**γ-Tocotrienol Inhibition of +SA Cell Proliferation.** Untreated control +SA cells displayed a progressive increase in viable cell number over the 3-day culture period (Fig. 1). In contrast, treatment with 1–8 μ*M* γ-tocotrienol resulted in a dose-responsive decrease in viable cell number over an identical time of incubation compared with that of untreated control cells (Fig. 1). From these studies it was determined that 4 μ*M* γ-tocotrienol caused an approximate 60% reduction in neoplastic +SA mammary epithelial cell growth, and this treatment dose was then used in subsequent studies to investigate the intracellular mechanisms involved in mediating the antiproliferative effects of γ-tocotrienol in these cells.

**γ-Tocotrienol Effects on PCNA Expression.** PCNA, a nuclear protein that displays elevated expression during S, G2, and M phases of the cell cycle, was used as a cellular marker for active cell proliferation (28). Positive PCNA staining in +SA cells is characterized by dark brown nuclear staining, whereas nonproliferating cells are characterized by light blue hematoxylin counterstaining. Prior to γ-tocotrienol treatment, nearly all +SA cells growing in culture displayed positive PCNA nuclear staining (Fig. 2A, Day 0). Following treatment with 4 μ*M* γ-tocotrienol, +SA cells displayed a time-dependent decrease in positive PCNA nuclear staining compared with untreated control cells (Fig. 2, Days 0–3). Quantification of 4 μ*M* γ-tocotrienol effects on +SA cell positive PCNA nuclear staining during the 3-day treatment in Figure 2A are shown in Figure 2B.

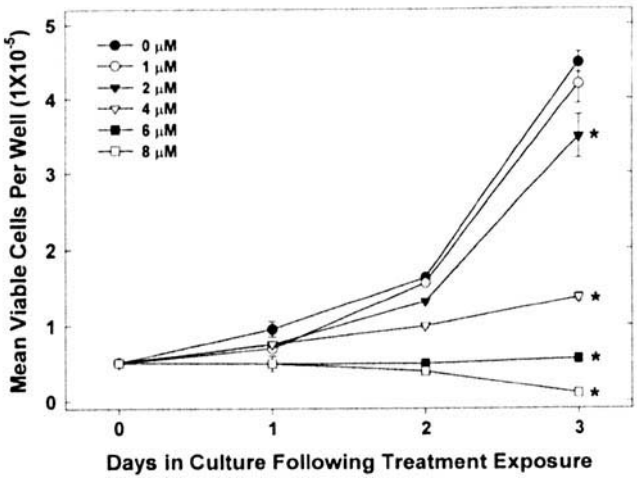


**Figure 1.** Effects of 0–8 μ*M* γ-tocotrienol on +SA cell proliferation during a 3-day culture period. Prior to treatment exposure, cells were plated at a density of $5 \times 10^4$ cells/well (6 wells/group). Data points indicate the mean viable cells/well ± SEM in each treatment group. *$P < 0.05$ compared with untreated control cells.

**γ-Tocotrienol Effects on Regulatory Proteins Associated with Akt and NFκB Signaling Pathways.** Figure 3 shows Western blot and scanning densitometric analyses of various intracellular proteins associated with Akt and NFκB mitogenic signaling. Western blot analysis showed that treatment with 4 μ*M* γ-tocotrienol caused a decrease in phospho-PDK1 (active) levels within 3 days after treatment exposure. Furthermore, while this same treatment did not alter total Akt levels (active and inactive combined), it did cause a large relative decrease in phospho-Akt (active) levels within 2 days after treatment exposure (Fig. 3). The effects of 4 μ*M* γ-tocotrienol on Akt kinase activity, as determined by phospho-GSK-3α/β protein levels, are shown in Figure 3. Because activated Akt exclusively phosphorylates glycogen synthase kinase (GSK)-3α/β, the relative intensity of the phospho-GSK-3α/β protein band in the Western blot is directly related to Akt kinase activity (29). Western blot analysis showed that treatment with 4 μ*M* γ-tocotrienol induced a large decrease in the relative levels of phospho-GSK-3α/β protein beginning 2 days after treatment exposure and indicates a decrease in Akt kinase activity (Fig. 3). Additional studies showed that treatment with 4 μ*M* tocotrienol does not affect total PTEN (active and inactive combined), phospho-PTEN (inactive), or PP2A catalytic subunit (active) levels in +SA cells, indicating that the decrease in phospho-Akt kinase activity is not due to an increase in activity of PTEN or PP2A phosphatase activity (Fig. 3). Furthermore, treatment with 4 μ*M* γ-tocotrienol also caused a relatively large decrease in levels of phospho-IKK-α/β (active), phospho-IκB-α (inactive), and phospho-NFκB p65 subunit (active) (Fig. 3). Because NFκB is downstream of IKK-α/β, these data indicate that γ-tocotrienol inhibition of NFkB activity occurs at the levels of IKK.

**γ-Tocotrienol Inhibition of p65 NFκB Transcriptional Activity.** The effects of 4 μ*M* γ-tocotrienol treatment on the transcriptional activity of p65 subunit of NFκB following a 3-day treatment exposure are shown in Figure 4. NFκB exists in an inactive state in the cytoplasm, bound to its inhibitory protein IκB, which prevents NFκB activation and translocation to the nucleus (20). Figure 4 shows that after a 3-day treatment period, NFκB activity was significantly reduced by approximately 65% in +SA cells treated with 4 μ*M* γ-tocotrienol, compared with that of untreated control cells.

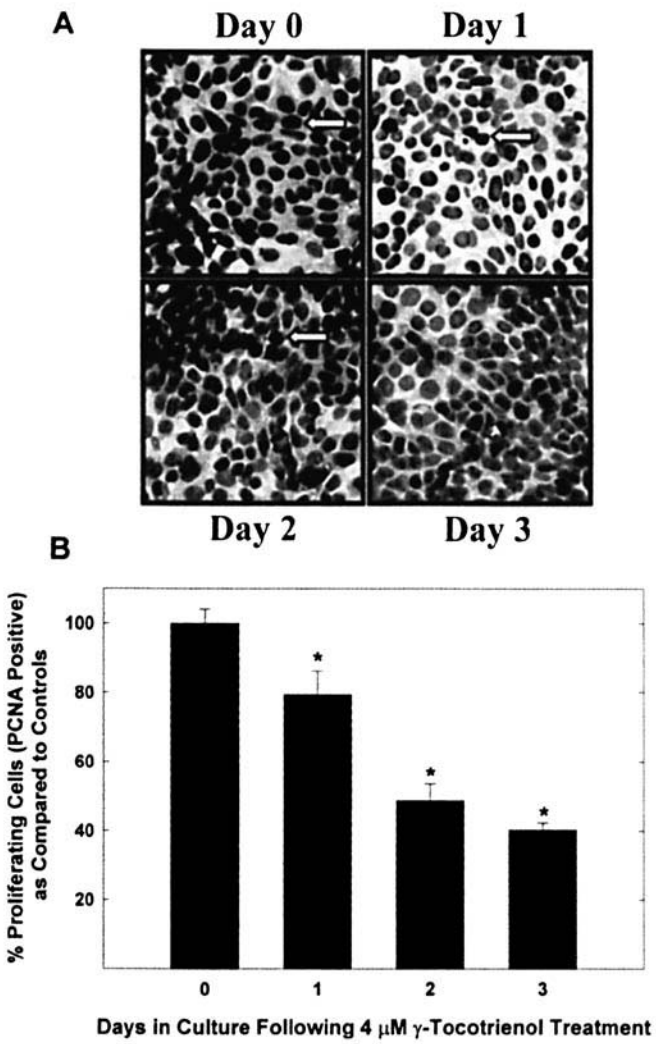


**Figure 2.** (A) Positive proliferating cell nuclear antigen (PCNA) immunocytochemical staining in +SA cells treated with 4 μ*M* γ-tocotrienol for 0–3 days. Cells were fixed with 4% paraformaldehyde, rinsed, and endogenous peroxidase activity was quenched by treatment with $H_2O_2$, as described in the PCNA kit instructions before PCNA assay. Arrows indicate positive PCNA staining. Magnification for each photomicrograph was ×200. (B) Percentage of +SA mammary epithelial cells displaying positive PCNA staining in each treatment group compared with untreated controls as shown in (A). The percentage of proliferating +SA cells was determined by counting the number of +SA cells displaying positive PCNA staining versus the total number of cells present in each photomicrograph for each treatment group. Cells were counted in five photomicrographs (80,000 $\mu m^2$ area per photomicrograph) for each treatment group. Vertical bars indicate the mean ± SEM. $^*P < 0.05$ compared with untreated control cells.

## Discussion

The results in this study demonstrate that the antiproliferative effects of γ-tocotrienol on neoplastic +SA mammary epithelial cells are associated with a reduction in EGF-dependent PI3K/PDK-1/Akt, and NFκB mitogenic signaling. Treatment with 4 μ*M* γ-tocotrienol caused a significant decrease in mitogen-dependent cell cycle progression, as indicated by a nearly 60% decrease in positive PCNA staining in these cells. This same treatment also caused a corresponding large decrease in intracellular levels of phospho-PDK-1 (active) and phospho-Akt (active) as well as Akt kinase activity, as indicated by a relatively large decrease in phospho-GSK-3α/β levels, a downstream substrate of activated Akt. Because PI3K and PDK-1 activations occur upstream and are a prerequisite for Akt activation, these findings strongly suggest that γ-tocotrienol may act to directly inhibit one or both of these kinases. This hypothesis is further evidenced by the finding that γ-tocotrienol treatment had no effect on the activity of PTEN and PP2A, phosphatases that are primarily responsible for regulating the dephosphorylation and inactivation of PDK-1 and Akt. In addition, the inhibitory effects of γ-tocotrienol on NFκB activation appear to result from the suppression of IKK-α/β activity, an upstream kinase that stimulates NFκB transcriptional activity.

Tocopherols and tocotrienols have previously been shown to modulate several intracellular signaling pathways involved with mitogenesis (30). Specifically, tocotrienol treatment has been found to inhibit EGF-dependent mitogenesis in normal, preneoplastic, and neoplastic mammary epithelial cells, whereas α-tocopherol does not (1–3, 5, 6). The mitogenic actions of EGF are mediated by specific membrane-bound receptors that have a cytoplasmic domain with intrinsic tyrosine kinase activity (8, 31). However, tocotrienol inhibition of EGF-dependent mitogenesis is not associated with a decrease in EGF-receptor levels or tyrosine kinase activity (2, 6). Therefore, the inhibitory effects of tocotrienols on EGF-dependent mitogenic signaling must occur downstream from the EGF-receptor. Several major signaling pathways have been shown to be associated with EGF-induced mitogenesis in neoplastic mammary epithelial cells, including the PI3K/PDK-1/Akt and NFκB signaling cascades (10, 11, 32, 33). PI3Ks represent a family of enzymes that are ubiquitously expressed and activated by numerous membrane receptors (15, 16). Activation of PI3K results in the 3′-phosphorylation of phosphoinositides within the cell membrane that bind and cause the translocation of Akt to the cell membrane, as well as effector proteins that act to transmit PI3K signaling downstream (15–17). PDK-1 is one of the best characterized membrane effectors of PI3K, which act to phosphorylate and activate Akt (13, 17, 18). Activation of Akt subsequently leads to the phosphorylation and regulation of various targets involved in cell survival (IκB, Bad, caspase-9, forkhead, and Daf transcription factors), mitogenesis (mTOR), and metabolism (GSK3-α/β) (13, 17, 18).

The present study shows that the antiproliferative effects of γ-tocotrienol are associated with a decrease in Akt phosphorylation at the Ser473 position, and a decrease in the phosphorylation and activity of Akt. Furthermore, tocotrienol-induced reductions in Akt phosphorylation do not result from an increase in PTEN or PP2A phosphatase activity. These findings suggest that the inhibitory effects of γ-tocotrienol on Akt activity result from direct inhibition of Akt or the inhibition of upstream kinases, such as PI3K, PDK-1, or both.

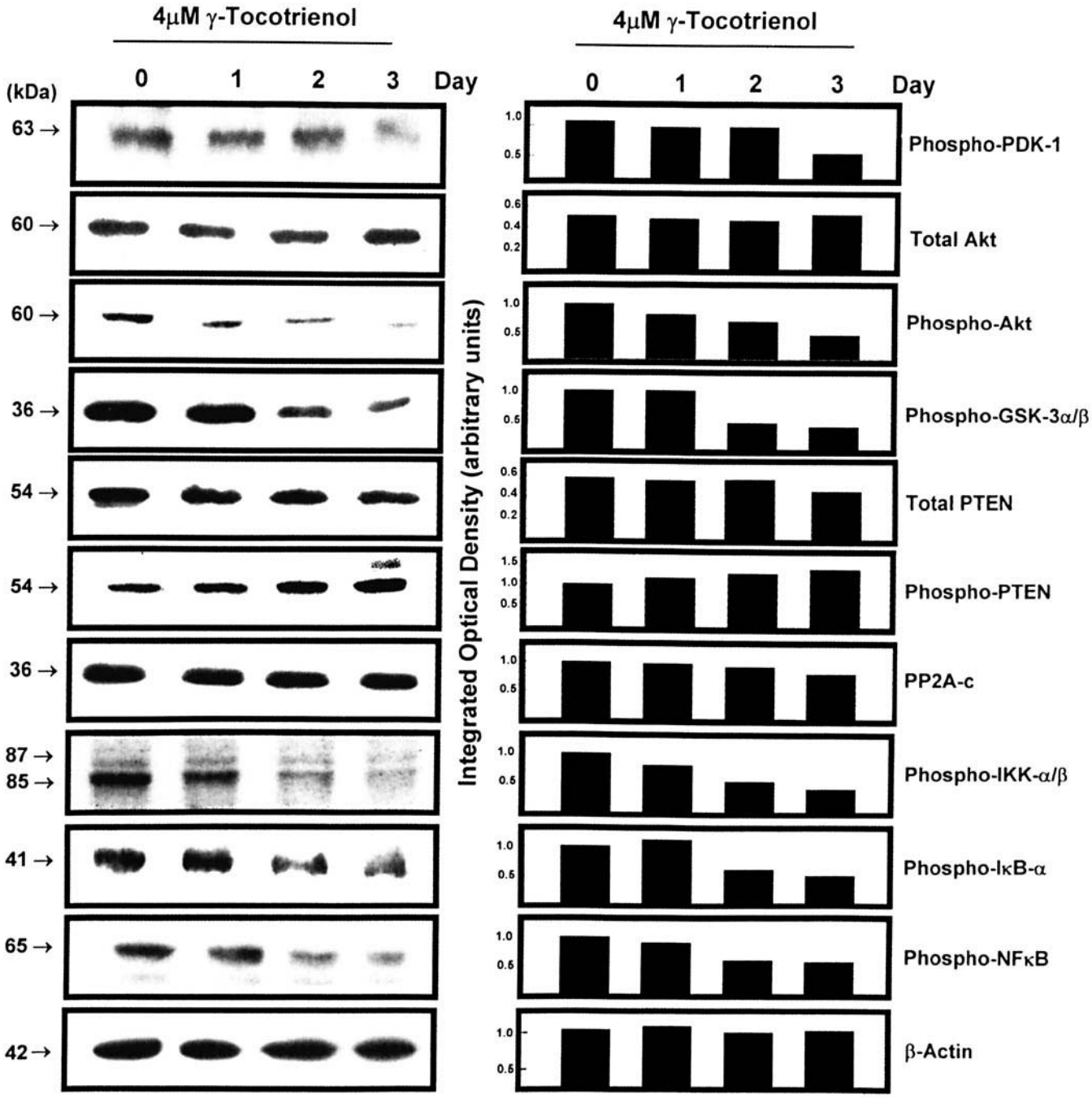


**Figure 3.** Western blot and scanning densitometric analyses of 4 μ*M* γ-tocotrienol treatment effects on the relative levels of intracellular proteins associated with phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase-1 (PDK-1)/Akt and NFκB mitogenic signaling pathways throughout a 3-day treatment period in neoplastic +SA mammary epithelial cells. Cells were collected each day, and whole cell lysates were prepared for subsequent separation by polyacrylamide gel electrophoresis (20 μg/lane), 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 before Western blot analysis for phospho-GSK-3-α/β. Each Western blot is a representative example of data obtained from experiments that were repeated at least three times. Scanning densitometric analysis was performed for each blot and is presented as bar graphs adjacent to its respective Western blot. Vertical bars in the densitometric graphs indicate the integrated optical density of bands visualized in each lane.

Tocopherol-associated proteins (TAPs) play a role in the intracellular trafficking of tocopherols (34). Recent studies have shown that TAPs also facilitate the transport of phosphatidylinositol (PI) to PI3K and enhance signaling along the PI3K/PDK-1/Akt pathway (34). In addition, tocopherols compete with PI for TAPs binding, and elevations in intracellular levels of tocopherols interfere with TAPs transport of PI to PI3K and can therefore potentially attenuate mitogen-dependent PI3K/PDK-1/Akt mitogenic signaling (34). Because neoplastic mammary epithelial cells preferentially take up and accumulate significantly larger concentrations of tocotrienols compared

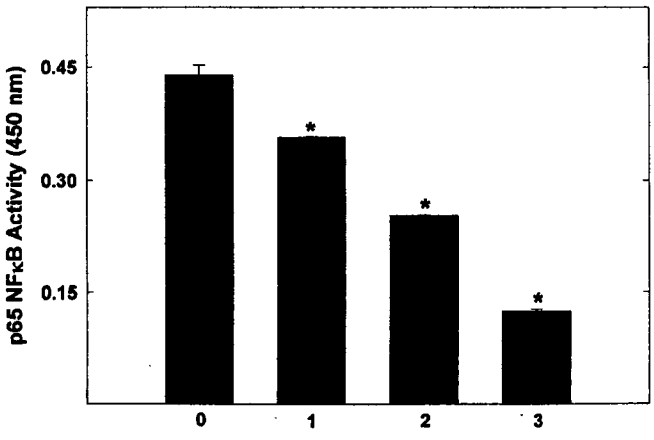


**Figure 4.** Effects of 4 μ*M* γ-tocotrienol treatment on NFκB (p65 subunit) transcriptional activity throughout the 3-day treatment period in neoplastic +SA mammary epithelial cells. Cells were collected each day, lysed, and then nuclear extracts were prepared for subsequent measurement of NFκB transcriptional activity. NFκB activity was measured per 10 μg of nuclear extract per sample in terms of optical density (OD) units at 450 nm. Vertical bars indicate the mean OD ± SEM for triplicates in each treatment group. $^*P < 0.05$ compared with untreated control cells.

with that of tocopherols (1, 5), it is possible the tocotrienols may also inhibit PI3K/PDK-1/Akt signaling by binding to TAPs and attenuating PI trafficking. However, additional studies are required to confirm this hypothesis and to determine the specific intracellular target site or sites of action of tocotrienols in neoplastic +SA mammary epithelial cells.

NFκB proteins constitute an inducible family of transcription factors that have been implicated in the regulation of cell proliferation, cell survival, tumor development, as well as malignant transformation (12, 14, 20, 22). Furthermore, EGF-induced activation of the PI3K/PDK-1/Akt mitogenic pathway has been shown to enhance NFκB transcriptional activity (11, 22). The NFκB family consists of five members (p50, p52, p65 [RelA], c-Rel, and RelB), which can form various homodimeric or heterodimeric complexes required for binding to DNA and initiating transcription (20). Elevations in NFκB activity is associated with enhanced tumor cell proliferation, survival, and metastatic potential (20). Classical NFκB is a heterodimer composed of the p65 and p50 subunits, and it is the p65 subunit that contains the transcriptional activation domain required for initiating gene transcription (20). NFκB proteins are sequestered in the cytoplasm in an inactive form when bound to the specific IκB inhibitory proteins such as IκB-α (20). A variety of mitogen-dependent kinases such as PI3K and Akt can activate the IKK complex to stimulate IκB degradation and NFκB activation (11, 19–21). Results in the present study provide evidence that inhibitory effects of γ-tocotrienol on NFκB activity most likely occur upstream at the level of IKK. Because NFκB is known to modulate the adhesive properties and promote the expression of a metastatic phenotype in malignant cells, these data also suggest that in addition to antiproliferative effects, γ-tocotrienol treatment may also act to reduce malignant cell metastatic potential. Additional studies are required to investigate this possibility.

In conclusion, the antiproliferative effects of γ-tocotrienol appear to be mediated, at least in part, through the suppression of PI3K/PDK-1/Akt and NFκB mitogenic signaling in neoplastic +SA mammary epithelial cells. Previous studies have shown that Akt and NFκB play a critical role in development and progression of mammary tumorigenesis, and overexpression of activated Akt and NFκB is associated with the development of resistance to chemotherapy in a majority of breast cancer cases (12–14, 19, 22, 35). If the present results can be reproduced in subsequent *in vivo* animal model studies, these findings would strongly suggest that tocotrienols may have potential value as a chemotherapeutic agent for use in the prevention or treatment of breast cancer in women.

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