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γ -Tocotrienol Inhibits Nuclear Factor- κ B Signaling Pathway through Inhibition of Receptor-interacting Protein and TAK1 Leading to Suppression of Antiapoptotic Gene Products and Potentiation of Apoptosis*

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Unlike the tocopherols, the tocotrienols, also members of the vitamin E family, have an unsaturated isoprenoid side chain. In contrast to extensive studies on tocopherol, very little is known about tocotrienol. Because the nuclear factor- κ B (NF- κ B) pathway has a central role in tumorigenesis, we investigated the effect of γ -tocotrienol on the NF- κ B pathway. Although γ -tocotrienol completely abolished tumor necrosis factor α (TNF)-induced NF- κ B activation, a similar dose of γ -tocopherol had no effect. Besides TNF, γ -tocotrienol also abolished NF- κ B activation induced by phorbol myristate acetate, okadaic acid, lipopolysaccharide, cigarette smoke, interleukin-1 β , and epidermal growth factor. Constitutive NF- κ B activation expressed by certain tumor cells was also abrogated by γ -tocotrienol. Reducing agent had no effect on the γ -tocotrienol-induced down-regulation of NF- κ B. Mevalonate reversed the NF- κ B inhibitory effect of γ -tocotrienol, indicating the role of hydroxymethylglutaryl-CoA reductase. γ -Tocotrienol blocked TNF-induced phosphorylation and degradation of I κ B α through the inhibition of I κ B α kinase activation, thus leading to the suppression of the phosphorylation and nuclear translocation of p65. γ -Tocotrienol also suppressed NF- κ B-dependent reporter gene transcription induced by TNF, TNFR1, TRADD, TRAF2, TAK1, receptor-interacting protein, NIK, and I κ B α kinase but not that activated by p65. Additionally, the expressions of NF- κ B-regulated gene products associated with antiapoptosis (IAP1, IAP2, Bcl-xL, Bcl-2, cFLIP, XIAP, Bfl-1/A1, TRAF1, and Survivin), proliferation (cyclin D1, COX2, and c-Myc), invasion (MMP-9 and ICAM-1), and angiogenesis (vascular endothelial growth factor) were down-regulated by γ -tocotrienol. This correlated with potentiation of apoptosis induced by TNF, paclitaxel, and doxorubicin. Overall, our results demonstrate that γ -tocotrienol inhibited the NF- κ B activation pathway, leading to down-regulation of various gene products and potentiation of apoptosis.

Although both tocopherols and tocotrienols exist as α , β , γ , and δ forms and are members of the vitamin E family, the two differ structurally in that tocopherols contain a saturated phytyl chain, and tocotrienols possess an unsaturated side chain. The source of these vitamins also differ; tocopherols are components of nuts and common vegetable oils and tocotrienols are primarily derived from oat, wheat germ, barley, rye, rice bran, and palm oil (1). The unsaturated side chain present in tocotrienols facilitates its entry through the membrane bilayer more efficiently than the saturated chain of tocopherol (2–4). Despite this advantage and some reports that tocotrienols are better antioxidants (1), there are 11,900 PubMed citations on tocopherol and less than 300 on tocotrienols.

Besides its activity against atherosclerosis (5–7), there are reports to suggest that tocotrienols may have potential against cancer. For instance, tocotrienols have been shown to suppress the proliferation of a wide variety of tumor cells in culture, including breast (8–12), prostate (13, 14), and colon (15). Animal studies have shown that tocotrienols can suppress the growth of breast tumor (16–18) and melanoma (19) and inhibit liver and lung carcinogenesis (20, 21).

Tocotrienols possess powerful neuroprotective, anti-cancer, and cholesterol-lowering properties that are often not exhibited by tocopherols (22). When mammary tumors were induced by 7,12-dimethylbenz(α)anthracene, only mice given tocotrienol had a significant increase in tumor latency; tocopherol had no effect (18). Similarly, only tocotrienol, not tocopherol, blocked the stress-induced changes in gastric acidity and gastrin level (23). Also, in contrast to tocotrienol, tocopherol showed very weak telomerase inhibition (15). A recent study has shown the ability of tocotrienols to preferentially sensitize prostate cancer to γ -radiation in nude mice (24).

How tocotrienols mediate their effects is not fully understood, but their abilities to induce cell cycle arrest (21), regulate HMG-CoA² reductase (25), activate p53 (26), activate caspase-8

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² The abbreviations used are: HMG-CoA, hydroxymethylglutaryl-CoA; TNF, tumor necrosis factor α ; NF- κ B, nuclear factor- κ B; I κ B, inhibitory subunit of NF- κ B; IKK, I κ B α kinase; SEAP, secretory alkaline phosphatase; PMA, phorbol myristate acetate; IAP, inhibitor of apoptosis protein; XIAP, X chromosome-linked IAP; FLIP, Fas-associated death domain protein-like interleukin-1 β -converting enzyme-inhibitory protein; COX, cyclooxygenase; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; TRAF, TNF receptor-associated factor; EMSA, electrophoretic mobility shift assay; LPS, lipo-

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(27), suppress adhesion molecules (28–30), down-regulate c-Myc and telomerase (15), and inhibit angiogenesis (31) have been established. Because of the critical role of NF- κ B pathway in tumorigenesis, radiosensitization, apoptosis, cell adhesion, expression of c-Myc and human telomerase reverse transcriptase, and cell cycle arrest (32), we postulated that γ -tocotrienol must modulate this pathway. The results described do indeed demonstrate that γ -tocotrienol can suppress NF- κ B activated by inflammatory cytokines, growth factors, and tumor promoters through the inhibition of I κ B α kinase, leading to suppression of NF- κ B-regulated gene products and potentiation of apoptosis.

MATERIALS AND METHODS

Reagents—Tocotrienols (α , β , γ , and δ) with a purity of 80, 20, 90, and 60%, respectively, were kindly supplied by W. H. Leong (Carotech, Inc., Edison, NJ). A solution of 80 mM α -tocotrienol (424 kDa; absorbance 292 nm using EM = 3870 is 3.106), 92 mM β -tocotrienol (410 kDa; absorbance 295 nm using EM = 3600 is 3.326), 66 mM γ -tocotrienol (410 kDa; absorbance 298 nm using EM = 4230 is 2.799), 98 mM δ -tocotrienol (396 kDa; absorbance 292 nm using EM = 3300 is 3.246), 100 mM γ -tocopherol (416 kDa; Sigma) was prepared in 100% dimethyl sulfoxide, stored as small aliquots at -20°C , and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate, prepared as described previously (33), was kindly supplied by Dr. C. G. Gairola (University of Kentucky, Lexington). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and FBS were obtained from Invitrogen. PMA, okadaic acid, LPS, IL-1 β , epidermal growth factor, and anti- β -actin antibody were obtained from Sigma. Antibodies against p65, p50, I κ B α , cyclin D1, MMP-9, PARP, IAP1, IAP2, TRAF1, Bcl-2, Bcl-xL, Bfl-1/A1, VEGF, c-Myc, Survivin, and ICAM-1 and the annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences. Phospho-specific anti-I κ B α (serine 32/36) and phospho-specific anti-p65 (serine 536) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-IKK- α , anti-IKK- β , and anti-cFLIP antibodies were kindly provided by Imgenex (San Diego). Expression vector plasmids for transforming growth factor- β -activated kinase (TAK1), TAK1-binding protein (TAB1), and receptor-interacting protein (RIP) were kindly provided by Dr. X. Lin (University of Texas, M. D. Anderson Cancer Center, Houston) (34).

Cell Lines—Human myeloid KBM-5 cells, human lung adenocarcinoma H1299 cells, human embryonic kidney A293 cells, human breast cancer MCF-7, multiple myeloma U266 cells, and human squamous cell carcinoma SCC-4 cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% FBS. H1299, MCF-7, and U266 cells were cul-

tured in RPMI 1640 medium, and A293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. SCC-4 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin.

Cytotoxicity Assay—The effect of γ -tocotrienol on the cytotoxic effects of TNF and chemotherapeutic reagents was determined by the MTT uptake method as described previously. Briefly, 5,000 cells were incubated with 5 μM γ -tocotrienol for 12 h in triplicate on 96-well plates and then treated with 5 nM paclitaxel and 100 nM doxorubicin for 24 h at 37°C . Thereafter, an MTT solution was added to each well. After 2 h of incubation at 37°C , extraction buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C , and the absorbance was then measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies, Chantilly, VA).

Live and Dead Assay—To assess cytotoxicity, we used the Live and Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. We performed this assay as described previously (35). Briefly, 2×10^5 cells were incubated with 25 μM γ -tocotrienol for 12 h and then treated with 1 nM TNF for 24 h at 37°C . Cells were stained with the Live and Dead reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan).

Annexin V Assay—One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of the cell to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin V. To detect apoptosis, we employed annexin V antibody conjugated with the fluorescent dye FITC. Briefly, 1×10^6 cells were pretreated with 25 μM γ -tocotrienol for 12 h, treated with 1 nM TNF for 24 h, and then subjected to annexin V staining. Cells were washed, stained with FITC-conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

Terminal Deoxynucleotidyltransferase-mediated Deoxyuridine Triphosphate Nick End-labeling Assay—We assayed cytotoxicity by the TUNEL method, which examines DNA strand breaks during apoptosis, using an *in situ* cell death detection reagent (Roche Applied Science). Briefly, 1×10^6 cells were pretreated with 25 μM γ -tocotrienol for 12 h and treated with 1 nM TNF for 24 h at 37°C . Thereafter, cells were incubated with reaction mixture for 60 min at 37°C . Stained cells were analyzed by flow cytometer (FACSCalibur, BD Biosciences).

PARP Cleavage Assay—For detection of cleavage products of PARP, whole-cell extracts were prepared by subjecting 25 μM γ -tocotrienol-treated cells to lysis in lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.01 $\mu\text{g/ml}$ aprotinin, 0.005 $\mu\text{g/ml}$ leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 4 mM NaVO_4). Lysates were spun at 14,000 rpm for 10 min to remove insoluble material, resolved by 7.5% SDS-PAGE, and probed with PARP antibodies.

Electrophoretic Mobility Shift Assay (EMSA)—To assess NF- κ B activation, we performed EMSA as described previously

polysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal dUTP nick-end labeling; PARP, poly(ADP-ribose) polymerase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; RIP, receptor-interacting protein.

(36). Briefly, nuclear extracts prepared from TNF-treated cells (2×10^6 /ml) were incubated with 32 P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide (15 μ g of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3' (boldface indicates NF- κ B-binding sites), for 30 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3', was used to examine the specificity of binding of NF- κ B to the DNA. The dried gels were visualized with a Storm820, and radioactive bands were quantitated using ImageQuant software (Amersham Biosciences).

Western Blot Analysis—To determine the levels of protein expression in the cytoplasm and nucleus, we prepared extracts (37) and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by ECL reagent (Amersham Biosciences). The bands were quantitated using a Personal Densitometer Scan version 1.30 using ImageQuant software version 3.3 (GE Healthcare).

IKK Assay—To determine the effect of γ -tocotrienol on TNF-induced IKK activation, IKK assay was performed by a method we described previously (38), with the following exceptions. Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKK- α and then treated with protein A/G-Sepharose beads (Pierce). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM MgCl₂, 2 mM dithiothreitol, 20 μ Ci of [γ - 32 P]ATP, 10 μ M unlabeled ATP, and 2 μ g of substrate GST-I κ B α (amino acids 1–54). After incubation at 30 °C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE; the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK- α and IKK- β in each sample, 50 μ g of whole-cell proteins was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK- α or anti-IKK- β antibody.

NF- κ B-dependent Reporter Gene Expression Assay—The effect of γ -tocotrienol on NF- κ B-dependent reporter gene transcription induced by TNF and various genes was analyzed by secretory alkaline phosphatase (SEAP) assay as described previously (39), with the following exceptions. Briefly, A293 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected by the calcium phosphate method with pNF- κ B-SEAP (0.5 μ g). To examine reporter gene expression induced by various genes, we transfected A293 cells with 0.5 μ g of pNF- κ B-SEAP plasmid with 1 μ g of an expressing plasmid and 0.5 μ g of the control plasmid pCMVFLAG1 for 24 h, treated them with 25 μ M γ -tocotrienol for 12 h, and then harvested them from culture medium after an additional 24 h of incubation. Culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech) using a Victor 3 microplate reader (PerkinElmer Life Sciences). For TAK1/TAB1 and RIP experi-

ments, A293 cells (5×10^5 cells/well) were transiently transfected with the expression vectors for TAK1/TAB1 or RIP and pNF- κ B-SEAP (0.5 μ g) plasmids by the FuGENE 6 method (Roche Applied Science). After 24 h, cells were treated with indicated concentrations of γ -tocotrienol, and conditioned medium was harvested after 12 h for SEAP activity. Cells were cotransfected with β -galactosidase and normalized the data with β -galactosidase assay (data not shown).

RESULTS

This study was designed to investigate the effects of γ -tocotrienol on the TNF-activated NF- κ B signaling pathway that regulates apoptosis. Most of the studies were carried out using human chronic myeloid leukemia cells (KBM-5) because these cells express both types of TNF receptors.

γ -Tocotrienol but Not γ -Tocopherol Blocks TNF-induced NF- κ B Activation—Because γ -tocopherol and γ -tocotrienol exhibit significant structural homology (Fig. 1A), we examined the effect of both types of vitamins for their ability to suppress TNF-induced NF- κ B activation. Fig. 1B shows that although neither of them alone activated NF- κ B, under identical conditions γ -tocotrienol completely suppressed TNF-induced NF- κ B activation, whereas γ -tocopherol did not suppress at all.

γ -Tocotrienol Blocks NF- κ B Activation Induced by Various Agents—Because various agents have been shown to activate NF- κ B through diverse pathways, we examined the effect of γ -tocotrienol on the NF- κ B activation induced by pro-inflammatory cytokines, growth factor, and carcinogens. A DNA-binding assay (EMSA) showed that γ -tocotrienol suppressed the NF- κ B activation induced by TNF, PMA, okadaic acid, cigarette smoke condensate, lipopolysaccharide, and epidermal growth factor (Fig. 1C). These results suggest that γ -tocotrienol acted at a step in the NF- κ B activation pathway that is common to all these agents.

Inhibition of NF- κ B Activation by γ -Tocotrienol Is Not Cell Type-specific—Distinct signal transduction pathways can mediate NF- κ B induction in different cell types (40, 41). Therefore, besides myeloid cells, we investigated the effect of γ -tocotrienol on TNF-induced NF- κ B activation in human epithelial H1299, A293, and MCF-7 cells (Fig. 1D). TNF activated NF- κ B in all these cells, and γ -tocotrienol suppressed this activation in all three cell lines.

γ -Tocotrienol Inhibits Constitutive NF- κ B Activation—A large variety of tumor cells express constitutively active NF- κ B, and this activation may differ from inducible activation (42). Therefore, we next tested the effect of γ -tocotrienol on NF- κ B activation in human multiple myeloma (U266) and head and neck squamous cell carcinoma (SCC4) tumor cells, which both express constitutively active NF- κ B (43, 44). U266 and SCC4 cells were treated with different concentrations of γ -tocotrienol and then analyzed for NF- κ B activation. γ -Tocotrienol inhibited constitutively active NF- κ B in both cell types in a dose-dependent manner (Fig. 1E). These results taken together indicate that γ -tocotrienol inhibited both inducible and constitutive NF- κ B activation.

γ -Tocotrienol Inhibits TNF-induced NF- κ B Activation in a Dose- and Time-dependent Manner—We next investigated whether suppression of TNF-induced NF- κ B by γ -tocotrienol is time-dependent. For this, we incubated the KBM-5 cells with 25

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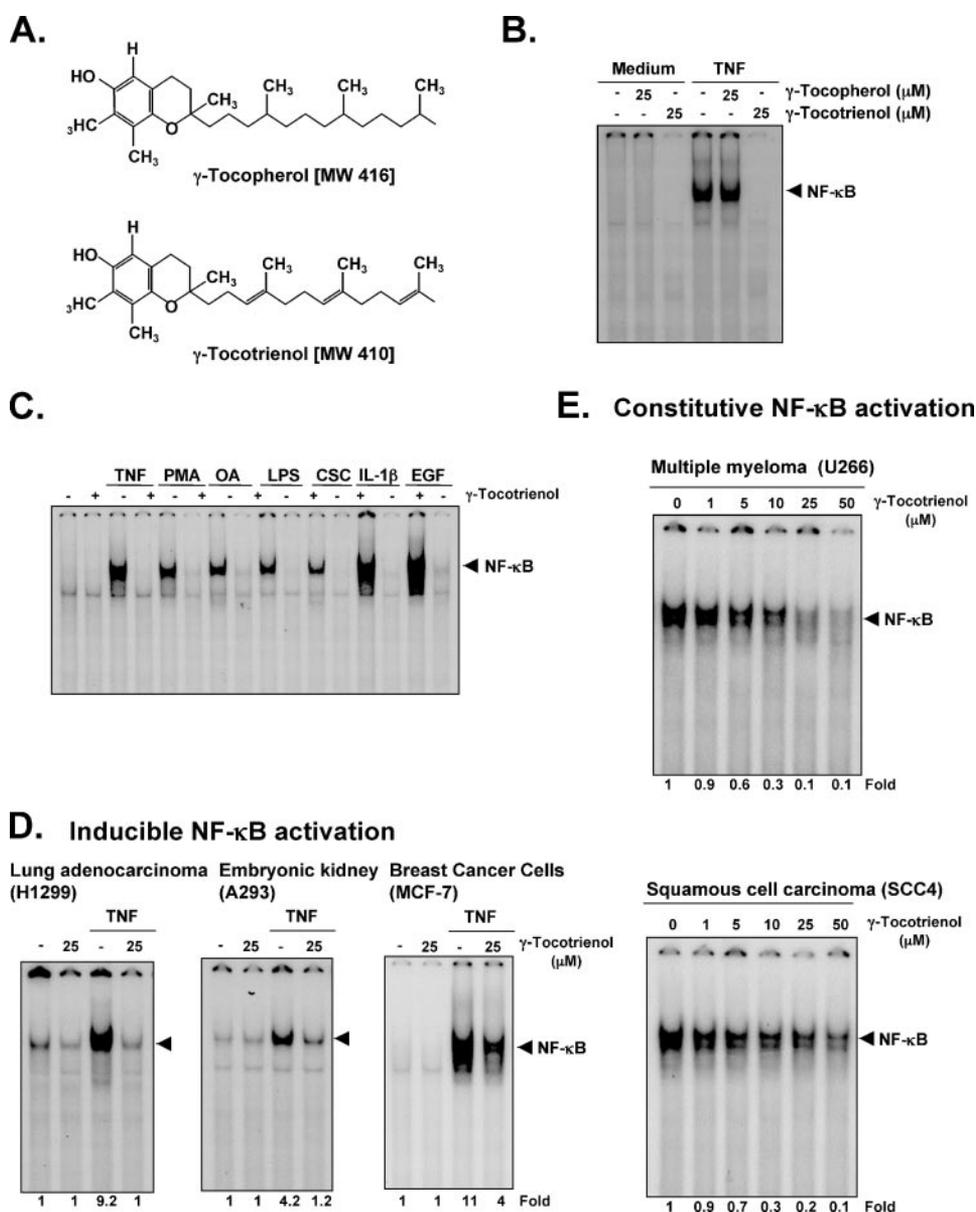


FIGURE 1. γ -Tocotrienol suppresses NF- κ B activation induced by proinflammatory cytokines, tumor promoters, carcinogen, and growth factor. A, structure of γ -tocopherol and γ -tocotrienol. B, KBM-5 cells were incubated with 25 μ M γ -tocopherol and γ -tocotrienol for 12 h and then with 0.1 nM TNF for 30 min. C, KBM-5 cells were incubated with 25 μ M γ -tocotrienol, 0.1 nM TNF for 30 min; with 25 ng/ml PMA, 100 ng/ml LPS and 100 nM IL-1 β for 1 h; 100 ng/ml epidermal growth factor (EGF) and 10 μ g/ml cigarette smoke condensate (CSC) for 2 h; and with 500 nM okadaic acid (OA) for 4 h, and then analyzed for NF- κ B activation by EMSA. D, human lung carcinoma H1299, human embryonic kidney A293, and human breast cancer MCF-7 cells were treated with 25 μ M γ -tocotrienol for 12 h and then with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA. E, constitutive NF- κ B activation in multiple myeloma U266 and squamous cell carcinoma SCC4 cells, which were incubated with the indicated concentrations of γ -tocotrienol for 12 h. Nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA.

μ M γ -tocotrienol for up to 24 h and then exposed the cells to 0.1 nM TNF for 30 min. EMSA results showed that γ -tocotrienol alone even for 24 h did not activate NF- κ B, but it abolished TNF-induced NF- κ B activation almost maximally at 12 h (Fig. 2A).

To determine the dose response, cells were pretreated with different doses of γ -tocotrienol for 12 h and then were stimulated with 0.1 nM TNF for 30 min. As indicated by EMSA, γ -tocotrienol suppressed TNF-induced NF- κ B activation in a dose-dependent manner (Fig. 2B). Under these conditions, cells were

fully viable as determined by the trypan blue dye exclusion test (data not shown).

To determine the effect of γ -tocotrienol on the time course of TNF-induced NF- κ B activation, we pretreated cells with 25 μ M γ -tocotrienol for 12 h, exposed them to 0.1 nM TNF for different times, and then examined the cells for NF- κ B in the nucleus by EMSA. The activation of NF- κ B by TNF was found to be time-dependent. Cells pretreated with γ -tocotrienol showed a lack of TNF-induced NF- κ B activation (Fig. 2C). To determine the effect of γ -tocotrienol on NF- κ B activation at higher TNF concentrations, cells were pretreated with 25 μ M γ -tocotrienol for 12 h, treated with various concentrations of TNF for 30 min, and then analyzed for NF- κ B activation (Fig. 2D). Cells pretreated with γ -tocotrienol showed a lack of NF- κ B activation even when exposed to a very high concentration of TNF. These results show that γ -tocotrienol is a very potent inhibitor of TNF-induced NF- κ B activation.

γ -Tocotrienol Does Not Interfere with the Binding of NF- κ B to DNA—Several agents have been described by our laboratory and others that modify the NF- κ B protein so that it can no longer bind to the DNA (45–48). Whether γ -tocotrienol mediates its effects through such a mechanism was examined. To determine this, we incubated nuclear extracts from TNF-induced cells with γ -tocotrienol and then analyzed DNA binding activity by EMSA. γ -Tocotrienol did not interfere with the DNA binding ability of the NF- κ B complex (Fig. 2E), indicating that γ -tocotrienol inhibited NF- κ B activation indirectly.

Glutathione Has No Effect on γ -Tocotrienol-induced Suppression of NF- κ B Activation—Several NF- κ B inhibitors that modulate NF- κ B by altering the redox status of the cells have been described (49–51). Whether γ -tocotrienol mediates its effects through a similar mechanism was examined. To determine this, cells were treated with γ -tocotrienol either in presence or absence of 100 μ M glutathione and then activated for NF- κ B. As shown in Fig. 2F, TNF-activated NF- κ B and γ -tocotrienol treatment suppressed the NF- κ B activation. Glutathione, however, had no effect on the γ -tocotrienol-induced suppression of TNF-induced NF- κ B activation.

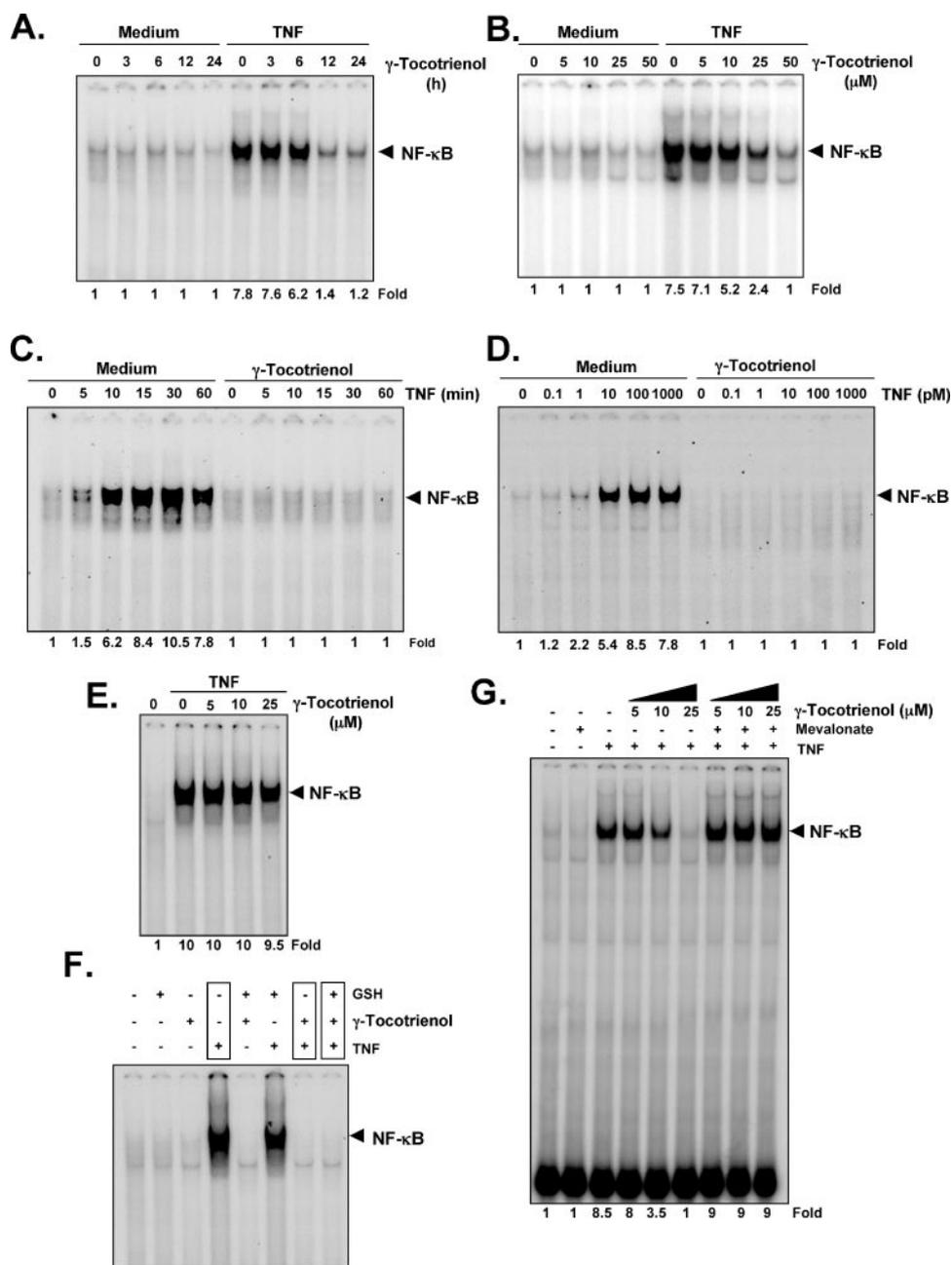


FIGURE 2. γ -Tocotrienol inhibits TNF-dependent NF- κ B activation. *A*, KBM-5 cells were preincubated with 25 μ M γ -tocotrienol for the indicated times, treated with 0.1 nM TNF for 30 min, and then subjected to EMSA for NF- κ B activation. *B*, cells were incubated with the indicated concentrations of γ -tocotrienol for 12 h and stimulated with 0.1 nM TNF for 30 min, and then nuclear extracts were subjected to EMSA for NF- κ B activation. *C*, cells were incubated with 25 μ M γ -tocotrienol for 12 h, treated with 0.1 nM TNF for the indicated times, and then analyzed for NF- κ B activation by EMSA. *D*, cells were incubated with 25 μ M γ -tocotrienol for 12 h, treated with indicated concentrations of TNF for 30 min, and then subjected to EMSA for NF- κ B activation. *E*, nuclear extracts from KBM-5 cells treated or not treated with 0.1 nM TNF for 30 min were treated with the indicated concentrations of γ -tocotrienol for 2 h at room temperature and then assayed for DNA binding by EMSA. *F*, cells were cotreated with 25 μ M γ -tocotrienol and 100 mM glutathione for 12 h, treated with 0.1 nM TNF for 30 min, and then subjected to EMSA for NF- κ B activation. *G*, KBM-5 cells were cotreated with 500 μ M L-mevalonate and γ -tocotrienol for 12 h and then stimulated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA.

L-Mevalonate Reverses γ -Tocotrienol-induced Suppression of NF- κ B Activation—We determined whether the inhibition of NF- κ B activation by γ -tocotrienol occurs through blocking HMG-CoA reductase activity. HMG-CoA catalyzes the conversion of HMG-CoA to mevalonate (52). We found that mevalonate reversed the inhibitory effect of γ -tocotrienol on TNF-

induced NF- κ B activation (Fig. 2*G*), suggesting that HMG-CoA reductase mediates the TNF-induced NF- κ B activation.

γ -Tocotrienol Inhibits TNF-dependent I κ B α Degradation—To determine whether the NF- κ B inhibitory activity of γ -tocotrienol was because of inhibition of I κ B α degradation, we pretreated cells with γ -tocotrienol, then exposed the cells to 0.1 nM TNF for different times, and examined the I κ B α status in the cytoplasm by Western blot analysis. TNF induced I κ B α degradation in control cells within 10 min and reached a maximum at 15 min, but TNF could not induce I κ B α degradation in γ -tocotrienol-pretreated cells (Fig. 3*A*). These results indicate that γ -tocotrienol inhibits TNF-induced I κ B α degradation.

γ -Tocotrienol Inhibits TNF-induced I κ B α Phosphorylation—Because I κ B α degradation requires its phosphorylation, we determined whether the inhibition of TNF-induced I κ B α degradation was because of inhibition of I κ B α phosphorylation. For this, we used the proteasome inhibitor using *N*-acetyl-leucylleucyl-norleucinal to block degradation of I κ B α (53). Cells were first treated with γ -tocotrienol and then with *N*-acetyl-leucylleucyl-norleucinal, exposed to TNF, and examined for I κ B α phosphorylation status using an antibody that recognizes the serine-phosphorylated form of I κ B α . Cells pretreated with the inhibitor showed a strong TNF-induced phosphorylation of I κ B α (Fig. 3*B*). γ -Tocotrienol significantly suppressed the I κ B α phosphorylation induced by TNF in the presence of the proteasome inhibitor (Fig. 3*B*).

γ -Tocotrienol Inhibits TNF-induced I κ B α Kinase Activation—IKK activation is required for TNF-induced phosphorylation of I κ B α

(41). Because γ -tocotrienol inhibits the phosphorylation of I κ B α , we determined the effect of γ -tocotrienol on TNF-induced IKK activation. The results from the immune complex kinase assay showed that TNF activated IKK as early as 3 min after TNF treatment, and γ -tocotrienol strongly suppressed this TNF-induced activation of IKK (Fig. 3*C*). As shown, TNF

γ -Tocotrienol Potentiates Apoptosis by Blocking NF- κ B Pathway

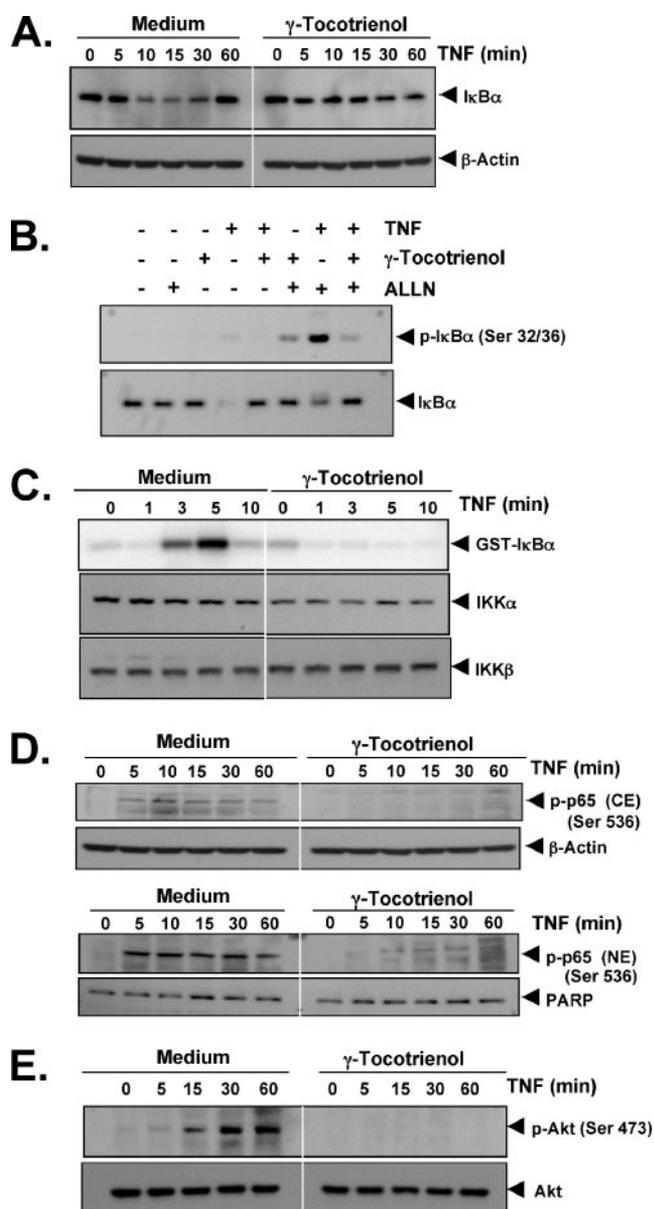


FIGURE 3. γ -Tocotrienol inhibits TNF-induced I κ B α degradation and I κ B α phosphorylation. *A*, KBM-5 cells were incubated with 25 μ M γ -tocotrienol for 12 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Western blot analysis was performed with anti-I κ B- α . *B*, cells were preincubated with 25 μ M γ -tocotrienol for 12 h, incubated with 50 μ g/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nM TNF for 15 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-I κ B α antibody. The same membrane was reblotted with anti-I κ B α antibody. *C*, γ -tocotrienol inhibits TNF-induced I κ B α kinase activity. KBM-5 cells were preincubated with 25 μ M γ -tocotrienol for 12 h, incubated with 50 μ g/ml ALLN for 30 min, and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKK- α and analyzed by an immune complex kinase assay. *D*, γ -tocotrienol inhibits TNF-dependent p65 phosphorylation. Cells were incubated with 25 μ M γ -tocotrienol for 12 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts (CE) and nuclear extracts (NE) were prepared, fractionated on SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using indicated antibodies. Anti- β -actin and anti-PARP antibodies were the loading control. *E*, γ -tocotrienol inhibits TNF-induced activation of Akt. KBM-5 cells were preincubated with 25 μ M γ -tocotrienol for 12 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-Akt antibody. The same membrane was reblotted with anti-Akt antibody.

or γ -tocotrienol or the two together had no direct effect on the expression of either IKK α or IKK β proteins.

γ -Tocotrienol Inhibits TNF-induced Phosphorylation of p65—Because the phosphorylation of p65 is required for its transcriptional activity (54), we tested the effect of γ -tocotrienol on TNF-induced phosphorylation of p65 in cytoplasm and nuclei. As shown by Western blot analysis in Fig. 3D, γ -tocotrienol abolished TNF-induced phosphorylation of cytoplasmic p65 completely. Likewise, TNF induced phosphorylation of nuclear p65 in a time-dependent manner, and γ -tocotrienol blocked its phosphorylation.

γ -Tocotrienol Inhibits TNF-induced Akt Activation—It has been reported that Akt can activate IKK (55). Akt activation has also been linked with phosphorylation of p65 (56). Thus, γ -tocotrienol may suppress TNF-induced Akt activation. To examine the effect of γ -tocotrienol on the TNF-induced activation of Akt, we pretreated cells with γ -tocotrienol and then exposed them to TNF, prepared whole-cell extracts, and performed Western blot analysis using antibody against the serine 473-phosphorylated form of Akt. TNF induced the Akt activation in a time-dependent manner, and γ -tocotrienol suppressed the activation completely with no significant effect on the expression of the Akt protein (Fig. 3E).

γ -Tocotrienol Represses TNF-induced NF- κ B-dependent Reporter Gene Expression—Although we investigated by EMSA that γ -tocotrienol inhibited NF- κ B activation, DNA binding alone does not always correlate with NF- κ B-dependent gene transcription, suggesting that there are additional regulatory steps (57). We next investigated where γ -tocotrienol acts in the sequence of TNFR1, TRADD, TRAF2, NIK, and IKK recruitment that characterizes TNF-induced NF- κ B activation (58, 59). In cells transfected with TNFR1, TRADD, NIK, IKK β , and p65 plasmids, NF- κ B-dependent gene expression was induced; γ -tocotrienol significantly suppressed reporter gene expression in all cells except those transfected with p65 (Fig. 4A). Because IKK activation can cause the phosphorylation of I κ B α and p65 (60), we suggest that γ -tocotrienol inhibits NF- κ B activation through inhibition of IKK.

γ -Tocotrienol Inhibits TAK1/TAB1-induced NF- κ B-dependent Reporter Gene Expression—TAK1, a member of the MAPK kinase kinase, was originally identified as a key regulator of MAPK activation in TGF- β -induced signaling pathways. It is activated by various inflammatory stimuli, including TNF, IL-1, and LPS (61). Recent studies indicate that TAK1 plays a major role in TNF-induced NF- κ B activation through its interaction with TAB1 and TAB2 (62). Therefore, we investigated whether γ -tocotrienol suppresses TNF-induced NF- κ B activation through the inhibition of TAK1. As shown in Fig. 4B, in cells transfected with TAK1/TAB1, NF- κ B-dependent reporter gene expression was induced, and γ -tocotrienol treatment inhibited this activation in a dose-dependent manner.

γ -Tocotrienol Inhibits RIP-induced NF- κ B-dependent Reporter Gene Expression—RIP is a serine/threonine kinase that plays an important role in TNF-induced NF- κ B activation (63, 64). RIP is ubiquitinated following TNF stimulation, which has been reported to play an important role in NF- κ B activation (65). Therefore, we investigated whether γ -tocotrienol suppresses TNF-induced NF- κ B activation through the inhibition

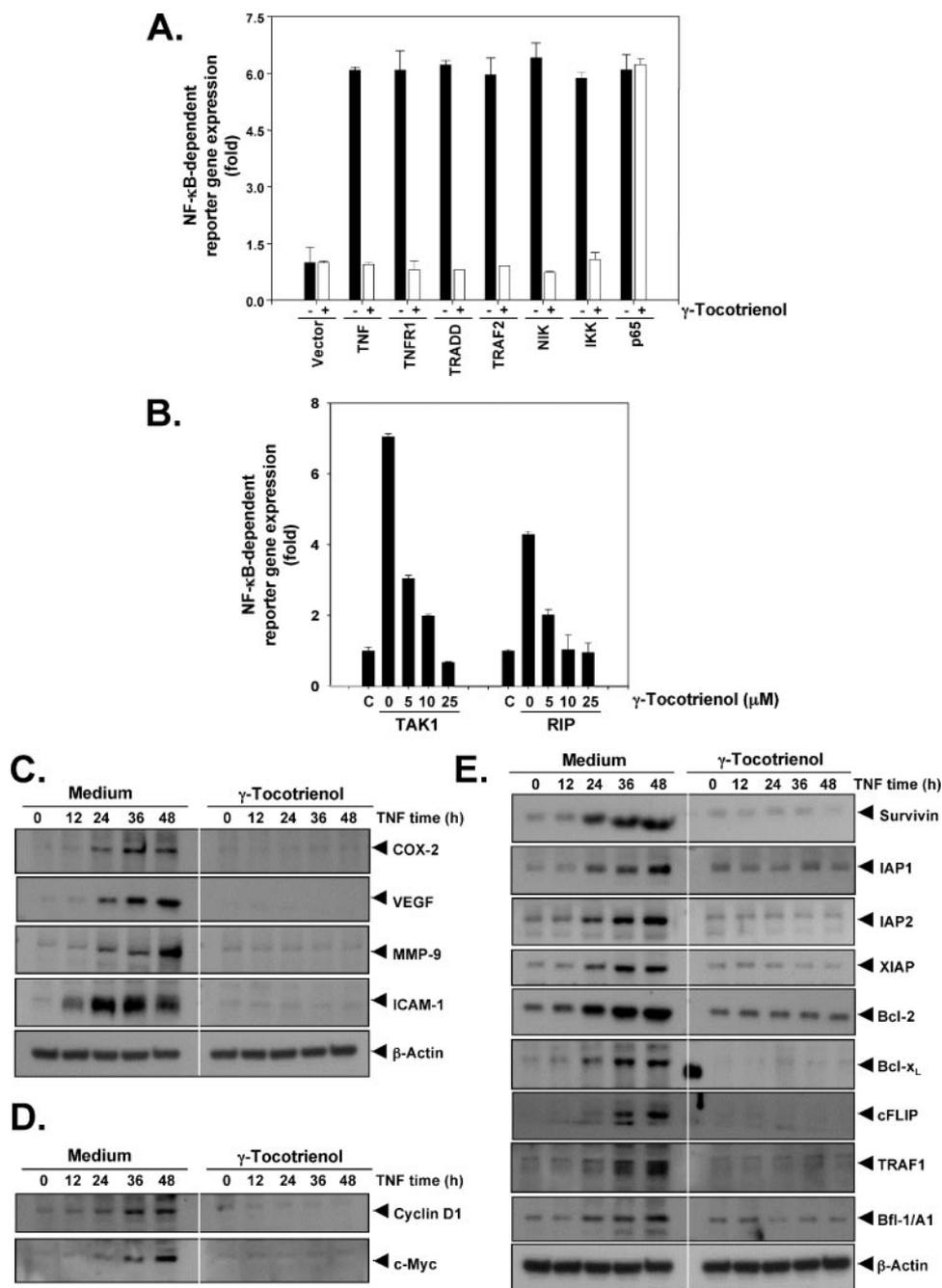


FIGURE 4. γ -Tocotrienol represses NF- κ B-dependent reporter gene expression induced by TNF and various plasmids. *A*, γ -tocotrienol inhibits the NF- κ B-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, IKK, and p65. Cells were transiently transfected with an NF- κ B-containing plasmid alone or with the indicated plasmids. After transfection, cells were incubated with 25 μ M γ -tocotrienol for 12 h and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were incubated with 25 μ M γ -tocotrienol for 12 h and then treated with 1 nM TNF for an additional 24 h of incubation. The supernatants of the culture medium were assayed for SEAP activity (data presented as means \pm S.D.). *B*, γ -tocotrienol suppresses TAK1/TAB1- and RIP-induced NF- κ B activation. A293 cells were transiently transfected with TAK1/TAB1 or RIP expression plasmids along with NF- κ B-containing plasmid. After 24 h, cells were treated with the indicated concentrations of γ -tocotrienol and incubated with the relevant plasmid for an additional 12 h. The supernatants of the culture medium were assayed for SEAP activity as described under "Materials and Methods." The results shown are representative of three independent experiments. *C*, γ -tocotrienol inhibits TNF-induced NF- κ B-regulated gene products. γ -Tocotrienol inhibits COX-2, VEGF, MMP-9, and ICAM-1 expression induced by TNF. KBM-5 cells (2×10^6 /ml) were left untreated or incubated with 25 μ M γ -tocotrienol for 12 h and then treated with 1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μ g of the whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, sliced from the membrane based on the molecular weight, and then probed with antibodies against VEGF, MMP-9, ICAM-1, COX-2, or β -actin, as described under "Materials and Methods." TNF-treated and TNF plus γ -tocotrienol-treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. *D*, γ -tocotrienol inhibits cyclin D1 and c-Myc expression induced by TNF. KBM-5 cells were left untreated or incubated with 25 μ M γ -tocotrienol for 12 h and then treated with 1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μ g of the whole-cell lysate was analyzed by Western blotting using antibodies against cyclin D1 and c-Myc. Data are for a representative experiment out of three independent ones showing similar results. TNF-treated and TNF plus γ -tocotrienol-treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. *E*, γ -tocotrienol inhibits the expression of antiapoptotic gene products IAP1, IAP2, Bcl-x_L, Bcl-2, XIAP, Bfl-1/A1, cFLIP, TRAF1, and Survivin. KBM-5 cells were left untreated or incubated with 25 μ M γ -tocotrienol for 12 h and then treated with 1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μ g of the whole-cell lysate was analyzed by Western blotting using antibodies against IAP1, IAP2, Bcl-x_L, Bcl-2, XIAP, Bfl-1/A1, cFLIP, TRAF1, and Survivin as indicated. TNF-treated and TNF plus γ -tocotrienol-treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. The results shown are representative of three independent experiments.

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of RIP. As shown in Fig. 4B, RIP activated NF- κ B reporter activity, and γ -tocotrienol suppressed the activation in a dose-dependent manner.

γ -Tocotrienol Suppresses TNF-induced NF- κ B-dependent Gene Products Involved in Cell Proliferation and Invasion—Numerous gene products, including COX-2, cyclin D1, VEGF, ICAM-1, MMP-9, and c-Myc, have an NF- κ B-binding site in their promoters (66–70). We investigated whether the expression of these gene products is modulated by γ -tocotrienol. Cells were pretreated with γ -tocotrienol for 12 h and then treated with TNF for the indicated times. Western blot analysis indicated that γ -tocotrienol suppressed the expression of these proteins (Fig. 4, C and D). The results provide further evidence of the role of γ -tocotrienol in blocking the expression of TNF-induced NF- κ B-regulated gene products.

γ -Tocotrienol Suppresses TNF-induced NF- κ B-dependent Antiapoptotic Gene Products—NF- κ B regulates the expression of several antiapoptotic proteins, including Survivin, IAP1, IAP2, Bcl-2, Bcl-xL, Bfl-1/A1, XIAP, TRAF1, and cFLIP (71–76). Whether γ -tocotrienol can modulate the expression of these antiapoptotic gene products induced by TNF was also examined. As shown in Fig. 4E, TNF induced the expression of these antiapoptotic proteins in a time-dependent manner, and γ -tocotrienol inhibited the expression.

γ -Tocotrienol Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agents—The NF- κ B activation inhibits apoptosis induced by TNF and chemotherapeutic agents through the regulation of gene products named above (77–81), so we investigated whether γ -tocotrienol can modulate the cytotoxic effects of TNF, paclitaxel, and doxorubicin. As indicated by the MTT method (Fig. 5A) and the Live and Dead assay (Fig. 5B), γ -tocotrienol up-regulated apoptotic effects of TNF and chemotherapeutic agents. The TNF-induced cytotoxicity was enhanced from 18 to 90% (Fig. 5B). The annexin V staining indicated that γ -tocotrienol up-regulated TNF-induced early apoptosis from 10 to 75% (Fig. 5C). TUNEL staining also indicated that apoptosis was enhanced by incubation with γ -tocotrienol from 1 to 92% (Fig. 5D). As shown in Fig. 5E, TNF-induced caspase activation, as indicated by PARP cleavage, was also potentiated by γ -tocotrienol. All these assays together suggest that γ -tocotrienol enhanced the apoptotic effects of TNF and chemotherapeutic agents.

γ -Tocotrienol Is the Most Potent Blocker of TNF-induced NF- κ B Activation among Tocotrienol Homologues—Tocotrienols exist as α , β , γ , and δ forms (Fig. 6A). Which form of tocotrienol homologues is most potent for its ability to suppress TNF-induced NF- κ B activation was investigated. We found that under these conditions γ -tocotrienol completely suppressed TNF-induced NF- κ B activation, and other forms were minimally active (Fig. 6B).

DISCUSSION

The goal of this study was to investigate the effect of γ -tocotrienol on the NF- κ B activation pathway and NF- κ B-regulated gene products that regulate apoptosis. We conclusively demonstrate that γ -tocotrienol suppressed NF- κ B activated by carcinogens, growth factors, and inflammatory stimuli through inhibition of IKK activation, I κ B α phosphorylation, I κ B α

degradation, p65 phosphorylation, and NF- κ B-dependent reporter gene expression. Under identical conditions, γ -tocopherol had no effect. As a result, γ -tocotrienol down-regulated the expression of NF- κ B-dependent gene products involved in cell proliferation, antiapoptosis, invasion and angiogenesis. We also demonstrated that γ -tocotrienol potentiated the apoptosis induced by TNF and chemotherapeutic agents (see Fig. 7).

This is the first report to investigate the effect of γ -tocotrienol on NF- κ B activation by a variety of stimuli. Our results show that γ -tocotrienol suppressed the NF- κ B activated by a variety of stimuli, suggesting that it must act at a step common to all these activators. Also, our results show that γ -tocotrienol blocked the activation of NF- κ B without directly interfering with the DNA binding of NF- κ B. NF- κ B activation in response to different stimuli requires IKK activation, which phosphorylates I κ B α at serines 32 and 36, leading to degradation of I κ B α (41). We found that this inhibition was mediated through the inhibition of IKK by γ -tocotrienol, which led to the suppression of phosphorylation and the degradation of I κ B α .

Besides inducible NF- κ B activation, we found that γ -tocotrienol also inhibited constitutive NF- κ B activation. Constitutively active NF- κ B has been found in a wide variety of leukemic and tumor epithelial cells (44), and it is needed for the proliferation of those cells (43, 82). The inhibition of the proliferation of neoplastic mammary epithelial cells by tocotrienols has been suggested to correlate with inhibition of NF- κ B activity (83). Why tumor cells express constitutively active NF- κ B is not fully understood, but the role of IKK has been implicated (43, 82). Thus it is possible that inhibition of IKK in tumor cells is linked to the ability of tocotrienol to suppress constitutive NF- κ B activation. Src-transformed fibroblasts have been shown to express constitutive NF- κ B (84). Because tocotrienols have been shown to inhibit c-Src (85), this may be another mechanism by which tocotrienols suppress constitutive NF- κ B activation.

Recently, several kinases have all been reported to function upstream of IKK, including Akt (54), NIK, MAPK1, GSK-3 β , PKR, SYK, atypical protein kinase C, and TAK1 (86). We found that γ -tocotrienol, among the IKK upstream regulatory kinases, inhibits TNF-induced Akt activation. These results agree with previous reports that showed that tocotrienol induces apoptosis in malignant epithelial cells by inhibiting phosphatidylinositol 3-kinase/PDK/Akt (11, 27). Akt has also been linked with the phosphorylation of p65 (60). Thus, it is possible that γ -tocotrienol inhibits TNF-induced IKK activation through the suppression of Akt activation. In addition to the suppression of Akt activation, different reports indicate that TAK1 plays a major role in TNF-induced NF- κ B activation through its interaction with TAB1 and TAB2. For instance, TAK1 can bind and activate IKK β leading to NF- κ B activation (87). TAK1 has also been shown to be recruited by the TNFR1 through TRADD, TRAF2, and RIP (34). Indeed, our study showed for the first time that TAK1-induced NF- κ B activation is inhibited by γ -tocotrienol. Whether γ -tocotrienol modulates other upstream kinases involved in NF- κ B activation cannot be excluded based on our studies. RIP also mediates the recruitment of TAK1 to TNF receptor complex and thus plays a very important role in

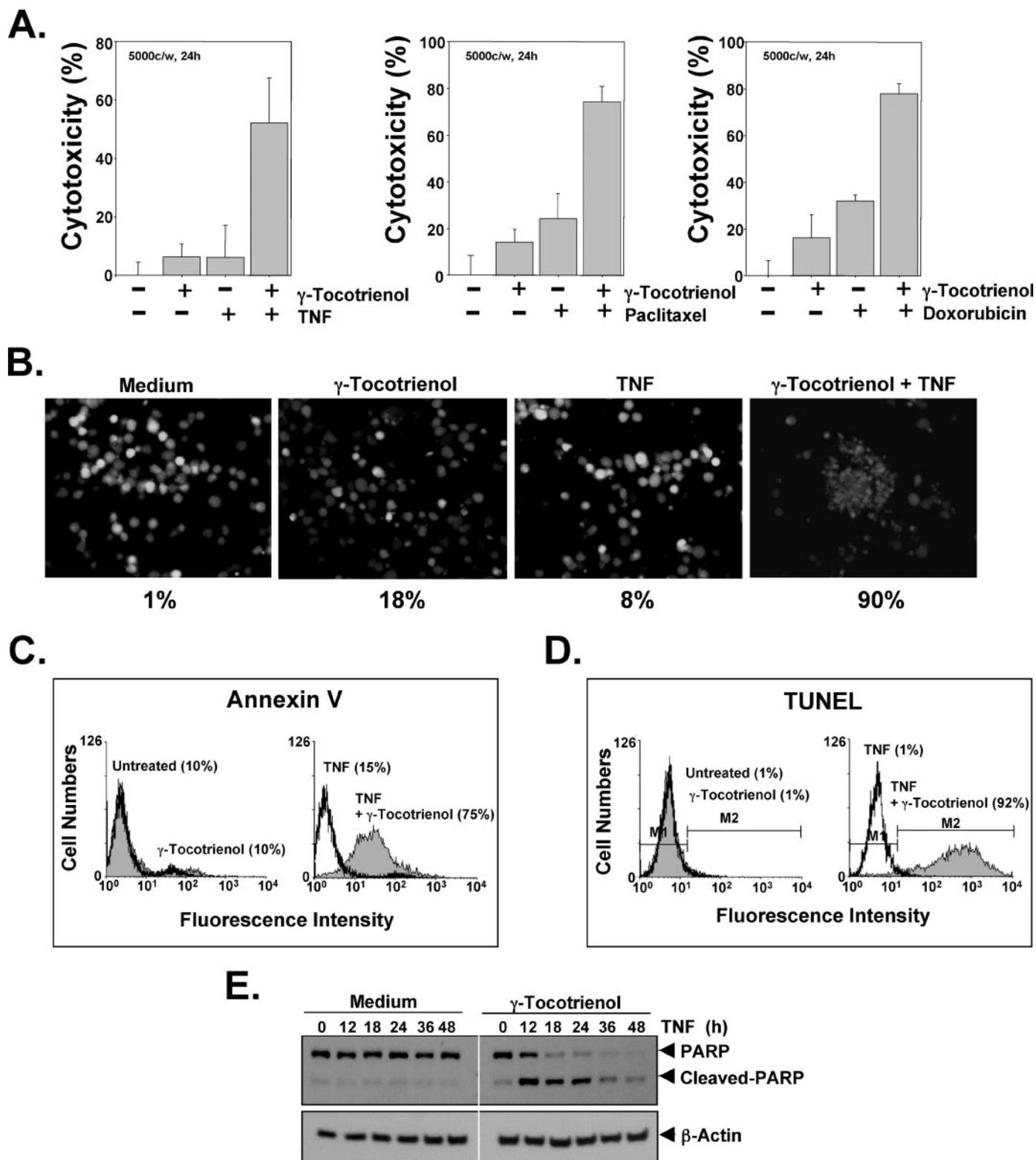


FIGURE 5. γ -Tocotrienol enhances apoptosis induced by TNF and chemotherapeutic agents. *A*, KBM-5 cells (5,000 cells/0.1 ml) were incubated at 37 °C with 1 nM TNF, 5 nM paclitaxel, or 100 nM doxorubicin in the presence and absence of 5 μ M γ -tocotrienol for 24 h, and the viable cells were assayed using the MTT reagent. The results are shown as the mean \pm S.D. from triplicate cultures. *B*, KBM-5 cells (1×10^6 /ml) were incubated with TNF, alone or in combination with 25 μ M γ -tocotrienol, as indicated for 24 h. Cell death was determined by the calcein-AM-based live/dead assay as described under "Materials and Methods." Red color highlights dead cells, and green color highlights live cells. *C*, cells were pretreated with 25 μ M γ -tocotrienol for 12 h and then incubated with 1 nM TNF for 24 h. Cells were incubated with anti-annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. *D*, KBM-5 cells were pretreated with 25 μ M γ -tocotrienol and 1 nM TNF for 24 h. Cells were fixed, stained with TUNEL assay reagent, and then analyzed by flow cytometer as described under "Materials and Methods." *E*, cells were pretreated with 25 μ M γ -tocotrienol for 12 h and then incubated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and subjected to Western blot analysis using anti-PARP antibody.

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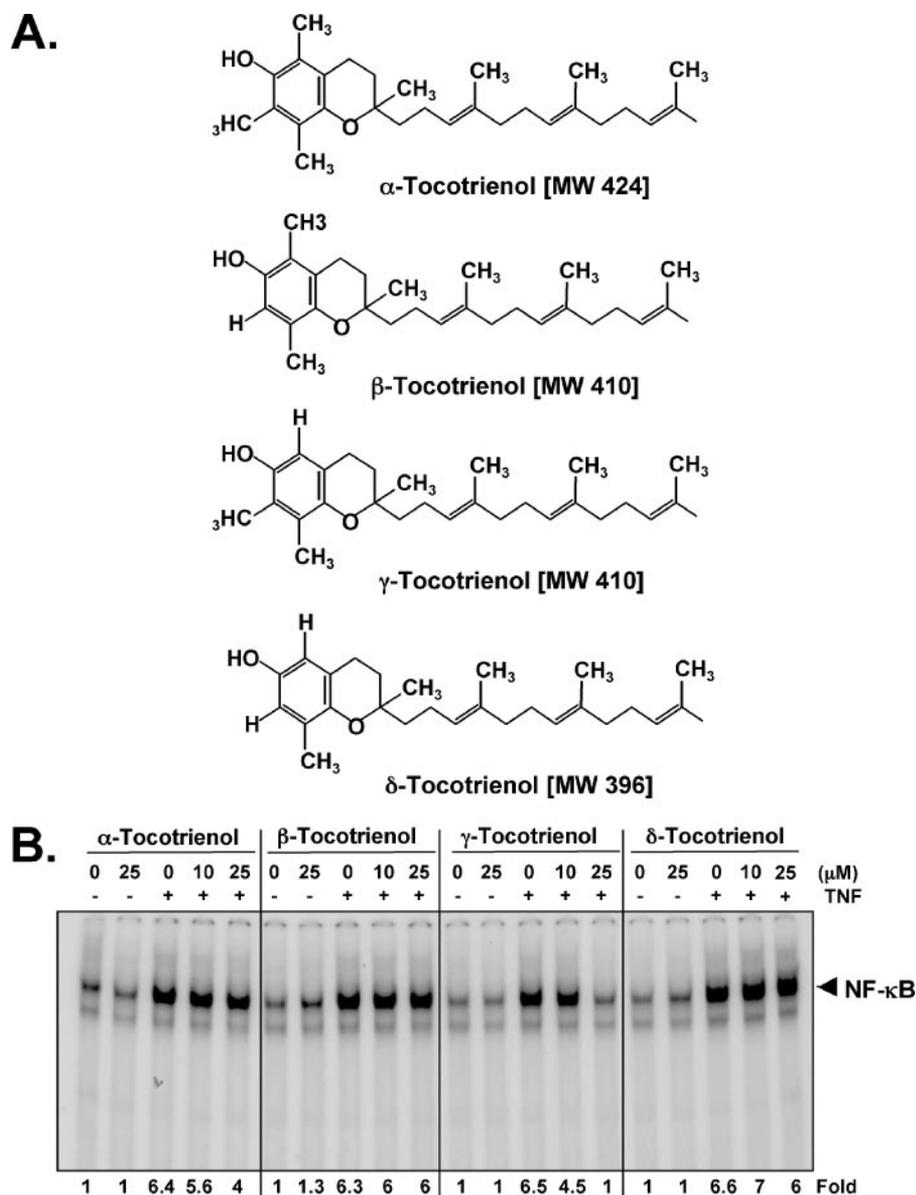


FIGURE 6. γ -Tocotrienol is the most potent blocker of TNF-induced NF- κ B activation. *A*, structures of tocotrienol homologues. *B*, KBM-5 cells were incubated with 10 and 25 μ M α -, β -, γ -, and δ -tocotrienol for 12 h and then stimulated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA.

TNF-induced IKK activation and subsequent NF- κ B activation (63, 65). We found that γ -tocotrienol inhibits RIP-induced NF- κ B-dependent reporter gene expression.

One of the mechanisms by which γ -tocotrienol could mediate its effects on the NF- κ B pathway is through its ability to induce the post-transcriptional suppression of HMG-CoA reductase that involves controlled degradation of the enzyme (25). Recent studies indicated that tocotrienols stimulate ubiquitination and degradation of the reductase (88). These results are consistent with a recent report from our laboratory that inhibitors of HMG-CoA reductase (statins) can suppress TNF-induced NF- κ B activation (89). Thus these results provide a link between the reductase that controls cholesterol biosynthesis and NF- κ B activation. Cardiovascular diseases linked with elevated cholesterol have been found to have constitutively active NF- κ B (90).

paclitaxel, and doxorubicin are enhanced by γ -tocotrienol. These effects suggested that γ -tocotrienol potentiates apoptosis by cytokine and chemotherapeutic agents.

We found that under the conditions in which γ -tocotrienol suppressed NF- κ B activation, γ -tocopherol had no effect. Our data are in agreement with other published reports that tocotrienol is a superior molecule among the members of the family of vitamin E (88, 92, 93). Four different homologues of tocotrienols have been identified, *viz.* α -, β -, γ -, and δ -. Under these conditions γ -tocotrienol completely suppressed TNF-induced NF- κ B activation, but other forms were found to have minimal effect. Published reports indicate that δ -tocotrienol is at least as active as γ -tocotrienol for inducing apoptosis (19, 93). The differences between our results and those reported previously could be due to the difference in purity of the tocotrienols employed or difference in the assays used.

We found that the expression of NF- κ B-regulated gene products involved in invasion (*e.g.* COX-2, MMP-9, and ICAM-1) and proliferation (*e.g.* cyclin D1 and c-Myc) were abrogated by γ -tocotrienol. Furthermore, our results showing that γ -tocotrienol suppresses NF- κ B-regulated COX-2 and ICAM-1 expression are in agreement with a previous report involving human neoplastic mammary epithelial cells and human umbilical vein endothelial cells (27, 29). Similarly, our findings that γ -tocotrienol down-regulates cyclin D1 expression are in agreement with previous reports (14) that showed that down-regulation of cyclin D1 is responsible for cell cycle arrest at the G₀/G₁ phase. Our report, however, is the first to show that γ -tocotrienol suppresses TNF-induced MMP-9 expression, which plays a crucial role in tumor invasion and angiogenesis by mediating the degradation of the extracellular matrix (91).

NF- κ B is known to regulate the expression of Survivin, IAPs, Bcl-2, Bcl-xL, Bfl-1/A1, cFLIP, and TRAF1, and their overexpression in numerous tumors has been associated with tumor survival, chemoresistance, and radioresistance. We showed that γ -tocotrienol down-regulates most of these gene products. These results are in agreement with recent findings that tocotrienol-rich fractions induce apoptosis through the modulation of Bax/Bcl-2 in colon carcinoma cells (26). The cytotoxic effects of TNF,

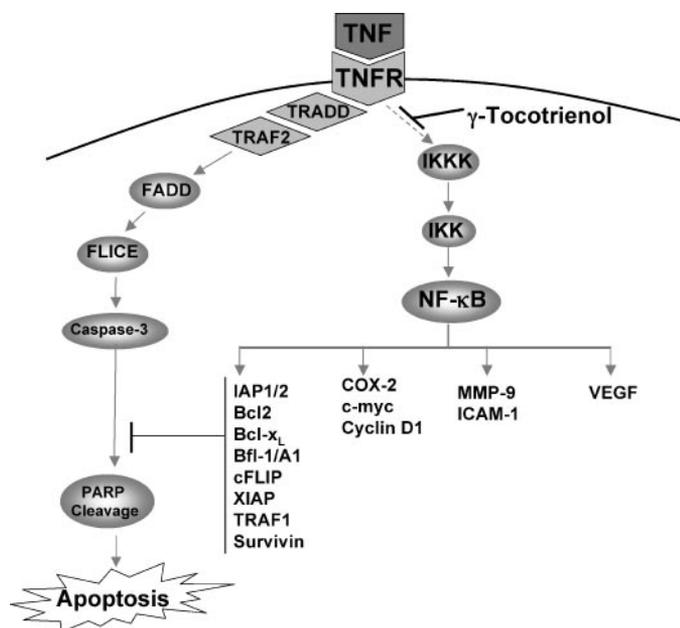


FIGURE 7. Schematic diagram showing the effect of γ -tocotrienol on NF- κ B signaling pathway and apoptosis.

Overall, our results demonstrate that γ -tocotrienol is a potent inhibitor of NF- κ B activation, which may explain its antiangiogenic, antiproliferative, proapoptotic, antimetastatic, anti-inflammatory, and immunomodulatory effects. Only 1% of all papers published on vitamin E have explored tocotrienols. Therefore, further studies are needed to explore its potential against cancer, cardiovascular, and neurological diseases.

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