Research Article

Dietary Administration of δ- and γ-Tocopherol Inhibits Tumorigenesis in the Animal Model of Estrogen Receptor–Positive, but not HER-2 Breast Cancer

Amanda K. Smolarek1,2, Jae Young So2, Brenda Burgess2, Ah-Ng Tony Kong2,5, Kenneth Reuhl1,5, Yong Lin4,5, Weichung Joe Shih4,5, Guangxun Li2, Mao-Jung Lee2, Yu-Kuo Chen2, Chung S. Yang2,5, and Nanjoo Suh2,5

University of New Jersey, Piscataway;4Department of Biostatistics, School of Chemical Biology and 3Pharmaceutics, Rutgers, The State University of New Jersey; and Nanjoo Suh, Department of Chemical Biology, State University of New Jersey, Piscataway, NJ 08854. Phone 732-445-

Introduction

Breast cancer is the most frequently diagnosed malignancy and a leading cause of cancer death among women (1). Lifestyle risk factors for breast cancer include obesity, lack of exercise, alcohol, and diet high in saturated fat (1, 2). A poor diet is estimated to be responsible for 15% to 35% of all cancer deaths (3). Vitamins and phytochemicals from fruits and vegetables may play a significant role in the prevention of cancer (4). Micronutrients may control intracellular events such as antioxidant activity, anti-inflammatory activity, and induction of apoptosis to reduce carcinogenesis (5).

Vitamin E is a fat-soluble antioxidant, which consists of 4 tocopherols and 4 tocotrienols and has been suggested to reduce cancer risk (6). Tocopherols have a saturated phytyl tail, whereas tocotrienols have an unsaturated isoprenoid side chain, which contains 3 double bonds (6). On the chromanol ring, the 4 variants (α-, β-, γ-, δ-) are determined by the number and position of methyl groups. Tocopherols are able to efficiently quench lipid-free radicals because of the phenolic group in the chromanol ring (7). Structural differences in the chromanol ring may be responsible for the variation in activity of each individual tocopherol form.

α-Tocopherol (trimethylated) is expected to be a more potent hydrogen donor and has greater antioxidant activity than either γ-tocopherol (dimethylated) or δ-tocopherol (monomethylated; refs. 8 and 9). However, γ- and δ-tocopherols lack a methyl group at the 5-position on the chromanol ring, and are more effective at trapping reactive nitrogen species than α-tocopherol (9).

α-Tocopherol is known as the classic vitamin E because of its important role in the fertility restoration assay (10).
However, α-tocopherol is not synonymous with vitamin E, and results from human intervention studies conducted with α-tocopherol are inconclusive. The Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study examined the prevention of lung and other cancers with supplementation of all-racemic-α-tocopherol acetate (50 mg/day) and β-carotene (20 mg/day) daily, and reported no effect of α-tocopherol supplementation on lung or colorectal cancer (11, 12). However, the ATBC study found that males supplemented with α-tocopherol had 32% lower prostate cancer incidence and 41% reduction in prostate cancer deaths (13). The Women’s Health Study (WHS) administered 600 IU of natural source vitamin E every other day for over 10 years. The WHS observed no overall benefit with supplementation in the prevention of cancer, but showed decreased cardiovascular mortality in healthy women (14). The Selenium and Vitamin E Cancer Prevention Trial (SELECT) using selenium (200 μg/day) and/or all racemic-α-tocopheryl acetate (400 IU/day) did not prevent prostate cancer with either agent alone or in combination (15). Interestingly, it was noted that the high dose of α-tocopherol decreased plasma γ-tocopherol levels and possibly limited cancer preventive and anti-inflammatory effects of γ-tocopherol (15). Previous intervention studies have used primarily α-tocopherol, not δ-tocopherol or γ-tocopherol, for chemoprevention (13–18). More research is needed on each form of tocopherol to determine their respective chemopreventive activity.

We have previously shown that administration of a diet containing 0.1%, 0.3%, or 0.5% of a γ-tocopherol rich mixture (γ-TmT) suppressed mammary tumor growth in N-methyl-N-nitrosourea (NMU)-induced rat model (19). In addition, we reported that 0.3% and 0.5% γ-TmT administered to August Copenhagen Irish rats with implanted estrogen pellets inhibited cell proliferation in mammary hyperplasia (20). In this study, we selected a single dose of 0.3% tocopherol in the diet to compare the efficacies of individual forms on 2 different animal models of mammary tumorigenesis. In the MMTV/ErbB2/neo transgenic mouse model overexpressing HER-2, tocopherols did not have long-term protective effects, whereas in the NMU-treated rat model, representing mainly estrogen receptor–positive breast cancer, δ- and γ-tocopherol, but not α-tocopherol, inhibited mammary growth.

Materials and Methods

Animals and experimental procedures

Female MMTV/ErbB2/neo transgenic mice 6 to 7 weeks old were purchased from Jackson Laboratory (Bar Harbor, ME). At 12 weeks of age, the mice received AIN-93M control diet or AIN-93M diets containing 0.3% tocopherols (α-, δ-, γ-, or γ-TmT; n = 28 per group). The body weight and tumor size of each animal were measured weekly. The mice were sacrificed at 55 weeks of age and the tumors were weighed at necropsy. Mammary glands, mammary tumors, and lungs were stored for further analyses. Serum was collected after centrifugation of clotted blood samples.

Female Sprague-Dawley rats were purchased from Tacornic Farms and were treated with a single intraperitoneal injection of the carcinogen NMU (50 mg/kg body weight) at 21 ± 1 days of age. One week after NMU injection, rats were fed AIN-93M control diet or AIN-93M diets containing 0.3% tocopherols (α-, δ-, γ-, or γ-TmT; n = 30 per group). Body weight and tumor volume were measured weekly. The rats were sacrificed 11 weeks after NMU injection. The mammary glands and mammary tumors were harvested, fixed in 10% formalin, and transferred to 70% ethanol or flash frozen and stored in −80°C. Blood was collected by cardiac puncture immediately before necropsy; serum was prepared and stored at −80°C. All animal studies were conducted in accordance with an institutionally approved protocol.

Diets

Semi-purified modified AIN-93M diet was obtained from Research Diets Laboratory and used as the control diet. The test diets were prepared by Research Diets Laboratory by adding 0.3% α-tocopherol, δ-tocopherol, γ-tocopherol, or γ-TmT to the AIN-93M diet. γ-TmT was supplied by the Cognis Corporation and contained 57% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol, and 1.5% β-tocopherol. γ-Tocopherol and γ-TmT were purified from γ-TmT by silica gel chromatography to a purity of 97%, with no detectable α-tocopherol and δ-tocopherol. δ-Tocopherol (containing 94% δ-tocopherol, 5.5% γ-tocopherol, and 0.5% α-tocopherol) and α-tocopherol (containing 69.7% α-tocopherol, 2.6% γ-tocopherol, and 0.2% δ-tocopherol) were purchased from Sigma-Aldrich. The diets were stored at 4°C and the food was replenished with fresh pellets twice weekly.

Serum estradiol levels

Estradiol (E2) levels in the serum were analyzed using an EIA kit from Enzo Life Sciences International, Inc. Serum samples were purified and the assay was conducted according to the manufacturer’s protocol.

Analysis of tocopherols in rat serum, mammary glands, and mammary tumors

Serum, mammary glands, and mammary tumors were analyzed by high-performance liquid chromatography for tocopherol (α, δ, γ) and short-chain metabolite, carboxyethyl hydroxychroman (CEHC) levels as previously described (19, 21).

Immunohistochemical analysis

Mammary glands and tumors were processed and stained as previously described (20). Sections were immunostained with antibodies to 8-hydroxy-2'-deoxyguanosine (8-oxo-dG; JaICA/GENOX Corporation), nitrotyrosine (Millipore), proliferating cell nuclear antigen (PCNA; BD Pharmingen), and cleaved-caspase 3 (c-Casp; Cell Signaling). Images were taken randomly with Nikon Eclipse E800 fitted to Nikon digital sight RI1. The staining density was determined by using an Aperio Scan Scope. Quantification was
done where 3 mammary glands or tumors from each treatment group were selected randomly and 3 areas from each gland or tumor were analyzed for over 1,000 cells per mammary gland or 4,000 cells per mammary tumor.

mRNA expression analysis using quantitative PCR
RNA was extracted from mammary tumors, and reverse transcription and quantitative PCR were carried out as previously reported (22). Labeled primers for glyceraldehyde-3-phosphate dehydrogenase, Bcl-2–associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), x-linked inhibitor of apoptosis (XIAP), PCNA, protein kinase C-α (PKC-α), phosphatase and tensin homologue (PTEN), Myc, p53, p21, p27, cyclin D1, estrogen receptor (ER)-α, ER-β, p21, p27, cyclin D1, estrogen receptor (ER)-α, ER-β, p21, p27, cyclin D1, estrogen receptor (ER)-α, and p53 were obtained from Applied Biosystems. The data presented represent the mean ± SE. The statistical significance was evaluated using ANOVA with Dunnett adjustment, preserving the overall type-I error at the 5% level. Tumor incidence was not significantly different among the groups (data not shown). After 7 weeks of treatment, the tumor incidence for the control group was 80% and similar in the α-tocopherol group (73.3%). However, the corresponding tumor incidence at week 7 for the δ-tocopherol, γ-tocopherol, and γ-TmT groups was markedly lower at 50.0% (P < 0.05), 56.7% (P = 0.057), and 53.3% (P < 0.05), respectively. At the conclusion of the 11-week study, the overall tumor incidence was not significantly different among the groups (Fig. 1B). As compared with the control group, dietary administration of δ-tocopherol and γ-tocopherol reduced tumor burden by 32% (P < 0.01) and 33% (P < 0.01), respectively, but α-tocopherol–enriched diet had no effect (Fig. 1B).

Western blot analysis
Mammary tumors were homogenized and the protein extracts were analyzed by Western blotting as previously described (19). The primary antibodies against apoptotic protease–activating factor 1 (Apaf-1), XIAP, cleaved-caspase-9 (c-Casp-9), cleaved-caspase-8 (c-Casp-8), cleaved-caspase-3 (c-Casp-3), cleaved-PARP (c-PARP), PKC-α, phospho-Akt (p-Akt), PTEN, p53, cyclin E, cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, TXN, and UGT were from Cell Signaling; Bcl-2, ER-α, PPAR-γ, c-Myc, p21, p27, cyclin D1, Nrf-2, KEAP1, and NQO1 were from Santa Cruz Biotechnology; Bax, SOD, GCLm, and GPx were from BD Pharmingen; GSTm1, catalase, and HO-1 were from Epitomics; and p53, ER-α, p21, p27, cyclin D1, estrogen receptor (ER)-α, and p53 were obtained from Cell Signaling. Tumor samples from different animals in each treatment group (n = 3 per group) were pooled for analysis by Western blot analysis.

Statistical analysis
Statistical significance was evaluated using ANOVA with Dunnett adjustment, preserving the overall type-I error at the 5% level. The primary antibodies against apoptotic protease–activating factor 1 (Apaf-1), XIAP, cleaved-caspase-9 (c-Casp-9), cleaved-caspase-8 (c-Casp-8), cleaved-caspase-3 (c-Casp-3), cleaved-PARP (c-PARP), PKC-α, phospho-Akt (p-Akt), PTEN, p53, cyclin E, cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, TXN, and UGT were from Cell Signaling; Bcl-2, ER-α, PPAR-γ, c-Myc, p21, p27, cyclin D1, Nrf-2, KEAP1, and NQO1 were from Santa Cruz Biotechnology; Bax, SOD, GCLm, and GPx were from Abcam; ER-β was from Affinity BioReagents; PCNA was from BD Pharmingen; GSTm1, catalase, and HO-1 were from Epitomics; and β-actin was from Sigma-Aldrich. Secondary antibodies were purchased from Santa Cruz Biotechnology. Tumor samples from different animals in each treatment group (n = 3 per group) were pooled for analysis by Western blot analysis.

Results
Dietary administration of tocopherols does not inhibit tumorigenesis in MMTV-ErbB2/neu transgenic mice
We investigated the effects of 0.3% α-tocopherol, 0.3% δ-tocopherol, 0.3% γ-tocopherol, or 0.3% γ-TmT in the diet on mammary tumor development in MMTV-ErbB2/neu transgenic mice. Body weight was measured weekly and there was no significant difference between treatment groups (data not shown). The median tumor latency was 37 weeks in the control group, and 38, 37, 44, and 39 weeks in mice fed with a diet containing α-tocopherol, δ-tocopherol, γ-tocopherol, and γ-TmT, respectively (Fig. 1A). Only the diet containing γ-tocopherol significantly increased the median tumor latency (P < 0.05). As shown in Fig. 1A, the final mammary tumor weight in the control group was 1.17 ± 0.14 g, compared with α-tocopherol (0.88 ± 0.11 g), δ-tocopherol (1.02 ± 0.09 g), γ-tocopherol (0.84 ± 0.11 g), and γ-TmT (1.11 ± 0.07 g), which corresponds to 24%, 13%, 29%, and 5% inhibition, respectively. The tumor multiplicity was 1.60 ± 0.30 in the control group, as compared with groups treated with α-tocopherol (1.18 ± 0.22), δ-tocopherol (1.32 ± 0.25), γ-tocopherol (1.28 ± 0.25), and γ-TmT (1.42 ± 0.28), which corresponds to 27%, 18%, 20%, and 11% inhibition, respectively (Fig. 1A). Although there was a delay with tumor incidence in the γ-tocopherol–fed group, all treatment groups were not effective in reducing the tumor weight at the time of sacrifice and no further analyses were conducted.

δ-Tocopherol and γ-tocopherol inhibit tumor growth and multiplicity in NMI-tREATED mammary tumorigenesis
Female Sprague-Dawley rats were either fed the control diet or the diets containing 0.3% α-tocopherol, 0.3% δ-tocopherol, 0.3% γ-tocopherol, or 0.3% γ-TmT. Body weight was measured weekly and there was no significant difference between treatment groups (data not shown). After 7 weeks of treatment, the tumor incidence for the control group was 80% and similar in the α-tocopherol group (73.3%). However, the corresponding tumor incidence at week 7 for the δ-tocopherol, γ-tocopherol, and γ-TmT groups was markedly lower at 50.0% (P < 0.05), 56.7% (P = 0.057), and 53.3% (P < 0.05), respectively. At the conclusion of the 11-week study, the overall tumor incidence was not significantly different among the groups (Fig. 1B). As compared with the control group, dietary administration of δ-tocopherol and γ-tocopherol reduced tumor burden by 32% (P < 0.01) and 33% (P < 0.01), respectively, but α-tocopherol–enriched diet had no effect (Fig. 1B). Tumor multiplicity for the control group was 5.0 ± 0.1 and was decreased by treatment with δ-tocopherol (2.9 ± 0.1), γ-tocopherol (3.4 ± 0.1), and γ-TmT (3.9 ± 0.1), which translates to 42% (P < 0.001), 32% (P < 0.01), and 22% (P < 0.05) inhibition, respectively (Fig. 1B). All subsequent serum and tissue analyses reported are for the activity of individual tocopherols in NMI-treated rats.

Serum levels of estradiol are decreased by the administration of tocopherols
Circulating endogenous serum levels of E2 were analyzed to determine changes between the treatment groups (Fig. 1B). Average E2 serum levels in the control group were 86.6
Inhibition of Mammary Carcinogenesis by Tocopherols

Levels of tocopherols and metabolites are increased in serum, mammary glands, and mammary tumors when treated with \( \alpha \)-tocopherol, \( \gamma \)-tocopherol, \( \delta \)-tocopherol, and \( \gamma \)-TmT diets

Serum, mammary gland, and mammary tumor samples were collected at necropsy and analyzed for the levels of \( \alpha \)-tocopherol, \( \gamma \)-tocopherol, \( \delta \)-tocopherol, and CEHC (Table 1). In general, the levels of individual tocopherols and CEHCs differed among serum, mammary glands, and mammary tumors. The highest levels of the hydrophobic parent tocopherols were found in the adipose-rich mammary gland, whereas comparable levels of tocopherols relative to control were found in both serum and mammary tumor. The water-soluble short chain metabolites were more prevalent in serum and mammary tumor compared with mammary gland.

Because of the selective transport of \( \alpha \)-tocopherol by the \( \alpha \)-tocopherol transport protein in the liver, \( \alpha \)-tocopherol is the major form found in serum. Levels of \( \alpha \)-tocopherol and \( \alpha \)-CEHC were significantly increased in serum, mammary glands, and mammary tumors of rats fed with 0.3% \( \alpha \)-tocopherol. Levels of \( \gamma \)-tocopherol and \( \gamma \)-CEHC were significantly increased in serum, mammary glands, and mammary tumors of rats fed with 0.3% \( \gamma \)-tocopherol; however, the levels of \( \alpha \)-tocopherol decreased in the serum \((P < 0.05)\), mammary glands \((P < 0.001)\), and mammary tumors \((P < 0.01)\) by treatment with \( \gamma \)-tocopherol. Administration of 0.3% \( \delta \)-tocopherol diet increased levels of \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \delta \)-CEHC in serum, mammary glands, and mammary tumors. Furthermore, 0.3% \( \gamma \)-TmT diet increased levels of \( \delta \)-tocopherol, \( \gamma \)-tocopherol, \( \alpha \)-CEHC, \( \delta \)-CEHC, and \( \gamma \)-CEHC in serum, mammary glands, and mammary tumors, whereas \( \alpha \)-tocopherol levels only increased in mammary glands.

Treatment with \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT induces apoptosis and inhibits cell proliferation and cell cycle in mammary tumors

As shown in Fig. 2A, the levels of proapoptotic proteins, Bax, c-Casp3, and c-PARP were increased by \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT–enriched diets. Furthermore, antiapoptotic proteins, Bcl-2 and XIAP, were inhibited by...
δ-tocopherol, γ-tocopherol, and γ-TmT. Levels of c-Casp9 were increased by all tocopherols, whereas c-Casp8 remained unchanged (data not shown). This may indicate that tocopherols induce apoptosis through the extrinsic apoptosis pathway.

The cell-cycle pathway plays a major role in regulating cancer development for the continuation of cell proliferation and survival. Cell proliferation markers, PCNA, and protein kinase C (PKC) were inhibited by treatment with δ-tocopherol, γ-tocopherol, and γ-TmT, but not by α-tocopherol (Fig. 2B). More specifically, in the cell survival pathway, PTEN was upregulated whereas p-Akt was downregulated by δ-tocopherol, γ-tocopherol, and γ-TmT. The oncogene cMyc regulates the G1 phase of the cell cycle; dietary δ-tocopherol and γ-tocopherol decreased cMyc protein levels in mammary tumors. Furthermore, protein levels of tumor suppressor p53, and CDK inhibitors p21 and p27 were increased by δ-tocopherol, γ-tocopherol, and γ-TmT. In contrast, α-tocopherol upregulated only p27. The protein level of cyclin D1 was decreased by γ-tocopherol and γ-TmT, CDK4 was reduced by δ-tocopherol, γ-tocopherol, and γ-TmT, and CDK6 was downregulated by each of the tocopherol-containing diets.

All tocopherol diets affected the Nrf2 pathway (Fig. 2C). Although levels of KEAP1 remained unchanged, the treatment with all tocopherols increased the protein levels of Nrf2. Furthermore, the levels of downstream phase II detoxifying and antioxidant enzymes TXN, GCLm, GSTM1, GPx, and HO-1 were increased by all tocopherols. NQO1 was induced by δ-tocopherol, γ-tocopherol, and γ-TmT, but not by α-tocopherol. Protein levels of SOD and UGT remained unchanged regardless of tocopherol treatment. Levels of nuclear receptors were examined in mammary tumors (Fig. 2D). Protein levels of ERα were decreased in samples from rats treated with α-tocopherol, δ-tocopherol, γ-tocopherol, and γ-TmT. Interestingly, the protein level of PPARγ was increased by δ-tocopherol, γ-tocopherol, and γ-TmT-containing diets, but not by α-tocopherol.

### Table 1. Analysis of tocopherol and short chain metabolite levels in NMU-treated rats fed with tocopherol (α-, δ-, γ-) and γ-TmT-containing diets

<table>
<thead>
<tr>
<th></th>
<th>Tocopherol</th>
<th>Short chain metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-T</td>
<td>δ-T</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.0 ± 1.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>0.3% α-T</td>
<td>72.8 ± 5.5***</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>0.3% δ-T</td>
<td>14.9 ± 1.3</td>
<td>10.6 ± 1.3***</td>
</tr>
<tr>
<td>0.3% γ-T</td>
<td>7.0 ± 0.6*</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>0.3% γ-TmT</td>
<td>23.1 ± 1.8</td>
<td>2.7 ± 0.3*</td>
</tr>
<tr>
<td><strong>Mammary gland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>147.9 ± 3.3</td>
<td>1.05 ± 0.3</td>
</tr>
<tr>
<td>0.3% α-T</td>
<td>215.7 ± 5.5***</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>0.3% δ-T</td>
<td>131.9 ± 7.5</td>
<td>131.3 ± 2.0***</td>
</tr>
<tr>
<td>0.3% γ-T</td>
<td>95.5 ± 4.3***</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>0.3% γ-TmT</td>
<td>171.3 ± 4.0*</td>
<td>96.5 ± 2.8***</td>
</tr>
<tr>
<td><strong>Tumor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 1.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0.3% α-T</td>
<td>19.6 ± 3.1***</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0.3% δ-T</td>
<td>6.0 ± 0.6</td>
<td>22.2 ± 1.3***</td>
</tr>
<tr>
<td>0.3% γ-T</td>
<td>2.0 ± 0.6**</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0.3% γ-TmT</td>
<td>11.8 ± 2.0</td>
<td>6.5 ± 0.8*</td>
</tr>
</tbody>
</table>

**NOTE:** The effects of 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT supplementation on the levels of tocopherols and their metabolites (CEHC) in serum (μmol/L), mammary gland (μmol/kg), and mammary tumor (μmol/kg) in NMU-treated rats. Data are presented as the mean ± SE (n = 6–12 per group).

*P < 0.05.

**P < 0.01.

***P < 0.001.
decreased by treatment with \( \delta \)-tocopherol (10%), \( \gamma \)-tocopherol (21%), and \( \gamma \)-TmT (14%); however, the results were not statistically significant (Fig. 3). Levels of 8-oxo-dG and nitrotyrosine were not reduced by \( \alpha \)-tocopherol in the mammary gland. In mammary tumors, markers of oxidative and nitrosative stress were markedly lower than mammary glands, and tocopherol treatment did not significantly change the levels of 8-oxo-dG and nitrotyrosine in mammary tumors.

Treatment with \( \delta \)-tocopherol, \( \gamma \)-tocopherol and \( \gamma \)-TmT reduces PCNA, and increases c-Casp3 in mammary tumors

In mammary glands, levels of PCNA and c-Casp3 did not change (data not shown). However, in mammary tumors, PCNA expression was reduced by \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT, but not by \( \alpha \)-tocopherol (Fig. 3). Administration of \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT resulted in a 24\% (\( P < 0.05 \)), 21\% (\( P < 0.05 \)), and 27\% (\( P < 0.05 \)) decrease in PCNA level, respectively. Furthermore, treatment by \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT increased c-Casp3 level in the mammary tumor by 89\% (\( P < 0.01 \)), 107\% (\( P < 0.01 \)), and 141\% (\( P < 0.001 \)) above the control group, respectively. \( \alpha \)-Tocopherol increased c-Casp3 level by 47\% above the control, but it was not statistically significant.

The mRNA levels for apoptotic, cell proliferation, cell survival, and cell-cycle markers, nuclear receptors, and Nrf2 pathways are regulated by tocopherols

The mRNA levels of apoptotic markers, Bax, Bcl-2, and XIAP were examined in mammary tumors (Table 2). The mRNA levels of Bcl-2 were decreased by \( \alpha \)-tocopherol (\( P < 0.01 \)), \( \delta \)-tocopherol (\( P < 0.05 \)), and \( \gamma \)-tocopherol (\( P < 0.05 \)), and \( \gamma \)-TmT (\( P < 0.01 \)), whereas BAX and XIAP were unchanged. Changes in mRNA levels of proliferation, survival, and cell-cycle pathway markers were examined in mammary tumors (Table 2). The tumor mRNA levels of

---

**Figure 2.** Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats associated with (A) apoptosis, (B) cell proliferation, survival, and cell cycle, (C) Nrf2 pathway, and (D) nuclear receptors. Mammary tumors were pooled together (\( n = 3 \) per group). Quantification of Western blot analysis was done by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot, respectively.
PTEN increased in rats treated with \( \alpha \)-tocopherol \((P < 0.05)\), \( \gamma \)-tocopherol \((P < 0.05)\), and \( \gamma \)-TmT \((P < 0.05)\), whereas \( \alpha \)-tocopherol did not. A CDK inhibitor, p21, was increased with \( \delta \)-tocopherol \((P < 0.05)\), \( \gamma \)-tocopherol \((P < 0.05)\), and \( \gamma \)-TmT \((P < 0.05)\) treatment; p27 was increased by \( \alpha \)-tocopherol \((P < 0.05)\), \( \delta \)-tocopherol \((P < 0.05)\), \( \gamma \)-tocopherol \((P < 0.01)\), and \( \gamma \)-TmT \((P < 0.001)\). However, the mRNA levels of PCNA, PKC\(\alpha\), Myc, p53, and cyclin D1 did not change by tocopherol treatment. mRNA levels for ER\(\alpha\) were significantly decreased by \( \delta \)-tocopherol \((P < 0.05)\) and \( \gamma \)-TmT \((P < 0.05)\) treatment; levels of ER\(\beta\) did not decrease. Interestingly, \( \delta \)-tocopherol, \( \gamma \)-tocopherol, and \( \gamma \)-TmT increased mRNA levels of PPAR\(\gamma\) \((P < 0.05, P < 0.01, P < 0.05)\) respectively; Table 2). Both Nrf2 and KEAP1 mRNA levels were unchanged by tocopherol treatment (Table 2). The phase II detoxifying enzymes GCLm, GSTm1, and Ugt1A1 were increased by all tocopherol treatment, whereas NQO1 and COMT mRNA levels were not affected. The mRNA levels of most of the antioxidant enzymes (SOD-1, HO-1, TXN1 and catalase) were unaltered.

**Discussion**

In this study, we examined the effects of individual tocopherols on 2 different animal models of breast cancer. The overexpression of ErbB2 has been reported in 18% to 25% of human breast cancers and is hormone independent (23). We found that \( \alpha \)-, \( \delta \)-tocopherol, and \( \gamma \)-TmT did not prevent the development of mammary tumorigenesis in transgenic MMTV/ErbB2/neu mice. At the conclusion of the 55-week study, tumor weight and multiplicity were not significantly affected by any of the examined tocopherols, suggesting that tocopherols do not affect HER-2–driven mammary tumorigenesis. There were modest effects by \( \gamma \)-tocopherol in reducing tumor incidence at 0.3% dose, and further studies may be needed to determine if higher doses of tocopherols are protective against HER-2 breast cancer. In our second

Figure 3. A, a representative immunostaining of nitrotyrosine, \( 8 \)-oxo-dG, PCNA, and c-Casp3 in the mammary gland or tumor of NMU-treated rats (600\( \times \)). Positive staining for nitrotyrosine is found in the cytoplasm of the cells. \( 8 \)-oxo-dG and PCNA show positive staining in the nuclei of the cells. Positive staining for c-Casp-3 is shown as a light brown to dark brown precipitate in the cytoplasm and or perinuclei of the cells. B, quantification was done using Aperio Scan Scope where 3 mammary glands or tumors from each treatment group were selected and 3 areas from each gland or tumor were analyzed for over 1,000 cells/mammary gland or 4,000 cells/mammary tumor. The data are presented as the mean \( \pm \) SE \((n = 3)\); *\( P < 0.05\), **\( P < 0.01\), ***\( P < 0.001\).
animal model, we used the carcinogen NMU to induce mammary tumors in female Sprague-Dawley rats. NMU-induced mammary tumors mainly represent estrogen-dependent and locally invasive phenotypes that are similar to human breast cancer (24, 25). Both δ- and γ-tocopherol, but not α-tocopherol, inhibited mammary tumor development and were examined further for molecular changes. Possible mechanisms of actions involved in the prevention of breast cancer by δ- and γ-tocopherol are represented in Fig. 4.

Tocopherols are known antioxidants and may reduce oxidative and nitrosative stress (RONS) to prevent cellular injury and mutations (26). RONS may promote tumor onset and progression by affecting DNA mutations, cell proliferation, and survival (27). γ-Tocopherol has been shown to be more effective at trapping reactive nitrogen species than α-tocopherol (9, 28–32). Previously, in a lung xenograft tumor model, δ-tocopherol, γ-tocopherol, and γ-TmT administration reduced 8-oxo-dG and nitrotyrosine levels, whereas α-tocopherol did not (33). In this study, treatment with δ-tocopherol, γ-tocopherol, and γ-TmT reduced 8-oxo-dG and nitrotyrosine levels in the mammary gland, whereas α-tocopherol did not. Interestingly, very low levels of RONS markers were observed in mammary tumors and were not changed by tocopherols. At the time of the analysis, the damage from the NMU carcinogen leading to

Table 2. Analysis of mRNA expression levels in the mammary tumor of NMU-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>α-T</th>
<th>δ-T</th>
<th>γ-T</th>
<th>γ-TmT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apoptotic markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Bcl2</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.1**</td>
<td>0.6 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
<td>0.4 ± 0.1**</td>
</tr>
<tr>
<td>XIAP</td>
<td>1.0 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td><strong>Cell proliferation, survival, and cycle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>PKCα</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>PTEN</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>2.0 ± 0.4*</td>
<td>2.1 ± 0.3*</td>
<td>1.6 ± 0.3*</td>
</tr>
<tr>
<td>Myc</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>p53</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>p21</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1*</td>
<td>1.4 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>p27</td>
<td>1.0 ± 0.3</td>
<td>1.8 ± 0.2*</td>
<td>2.0 ± 0.4*</td>
<td>2.1 ± 0.3**</td>
<td>2.5 ± 0.3***</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Nuclear receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-α</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.0*</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>ER-β</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.8 ± 0.2*</td>
<td>2.1 ± 0.2**</td>
<td>1.9 ± 0.1*</td>
</tr>
<tr>
<td>Nrf2 pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nrf2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Keap1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td><strong>Phase II detoxifying enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NQO1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>GCLm</td>
<td>1.0 ± 0.3</td>
<td>2.8 ± 0.9*</td>
<td>2.5 ± 0.2*</td>
<td>2.8 ± 0.5*</td>
<td>2.9 ± 0.4*</td>
</tr>
<tr>
<td>GSTm1</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 0.2*</td>
<td>1.5 ± 0.1*</td>
<td>1.7 ± 0.1*</td>
<td>2.0 ± 0.5*</td>
</tr>
<tr>
<td>Ugt1A1</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.3**</td>
<td>1.7 ± 0.2*</td>
<td>1.7 ± 0.2*</td>
<td>1.8 ± 0.4*</td>
</tr>
<tr>
<td>COMT</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td><strong>Antioxidant enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>HO-1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>GPx</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.2*</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>TXN1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

NOTE: NMU-treated Sprague-Dawley rats were administered 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT in the diet. A mammary tumor from each rat was analyzed for mRNA levels by quantitative PCR and normalized by GAPDH. The values (fold induction) are represented as mean ± SE (n = 6–8 per group).

*P < 0.05.
**P < 0.01.
***P < 0.001.
mammary tumorigenesis already occurred and may explain why we did not see changes in RONS levels in mammary tumors.

Tocopherols may also function as indirect antioxidants by stimulating the Nrf2 pathway. The downstream enzymes in the Nrf2 pathway protect cells from neoplastic transformation by maintaining oxidative stress homeostasis (34, 35). More importantly, the loss of Nrf-2 may lead to an increase in inflammation and to a decrease in cellular defense against oxidative stress, which may result in tumorigenesis (36). We reported that administration of γ-TmT increased protein levels of Nrf2 and mRNA levels of UGT1A1, GSTm1, and COMT in estrogen-induced mammary hyperplasia (20). In this study, we showed that all tocopherol treatments had similar effects in modulating Bcl-2, c-Casp-9, c-Casp-3, and c-PARP are increased, whereas antiapoptotic proteins XIAP and Bcl-2 are decreased by δ- and γ-tocopherol. In summary, δ- and γ-tocopherol may activate PPAR-γ, PTEN, p53, the apoptotic pathway, and Nrf-2 and decrease ER-α, p-Akt, cyclin D1, and PCNA to inhibit cell-cycle progression and cell growth resulting in the inhibition of mammary tumorigenesis.

α-Tocopherol has been the primary tocopherol used for chemoprevention studies, and the results have been inconclusive (6, 39, 40). Furthermore, the precise mechanism of action of individual tocopherols in cancer prevention is still unknown. We found that all tocopherol treatments had similar effects in modulating Bcl-2, c-Casp-9, c-PARP, ER-α, p27, CDK6, and the Nrf2 pathway (Fig. 2). However, δ- and γ-tocopherol lack a methyl group at the 5’ position on the chromanol ring whereas α-tocopherol does not. This structural difference may attribute to the efficacy to remove RNS. δ- and γ-tocopherol, but not α-tocopherol, reduced levels of nitrotyrosine in the mammary gland (Fig. 3). δ- and γ-tocopherol may delay tumor onset through the reduction on RNS. In mammary tumors, δ- and γ-tocopherol, but not α-tocopherol, increased levels of PTEN, p53 pathway, PPAR-γ, and c-Casp-3, whereas the levels of pAkt and PCNA decreased (Figs. 2 and 3). The relationship between ER-α, PPAR-γ, PTEN, Akt, and p53 may be an important aspect in determining the chemopreventive activity of tocopherols.
mechanism of action for the inhibition of mammary tumorigenesis by δ- and γ-tocopherol in vivo. Our findings indicate that δ- and γ-tocopherol, but not α-tocopherol, work through antioxidant-dependent pathway that decreases cell survival and proliferation, regulating cell cycle and inducing PPARγ and apoptosis, leading to the inhibition of mammary tumorigenesis. This suggests that δ- and γ-tocopherol, but not α-tocopherol, are useful in the prevention of hormone-dependent breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. K. Smolarek, J.-Y. So, Y. Lin, C. S. Yang, N. Suh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. K. Smolarek, J.-Y. So, K. Reuhl, N. Suh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. K. Smolarek, B. Burgess, K. Reuhl, Y. Lin, W. J. Shih, A.-N. Tony Kong, Y. Lin, M.-J. Lee, C. S. Yang, N. Suh
Writing, review, and/or revision of the manuscript: A. K. Smolarek, J.-Y. So, B. Burgess, A.-N. Tony Kong, K. Reuhl, Y. Lin, M.-J. Lee, C. S. Yang, N. Suh

References
Dietary Administration of δ- and γ-Tocopherol Inhibits Tumorigenesis in the Animal Model of Estrogen Receptor–Positive, but not HER-2 Breast Cancer

Amanda K. Smolarek, Jae Young So, Brenda Burgess, et al.


Updated version

Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-12-0263

Cited articles

This article cites 38 articles, 9 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/5/11/1310.full#ref-list-1

Citing articles

This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerpreventionresearch.aacrjournals.org/content/5/11/1310.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/5/11/1310. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.