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Int J Cancer. Author manuscript; available in PMC 2013 February 01.

Published in final edited form as:
Int J Cancer. 2012 February 1; 130(3): 685–693. doi:10.1002/ijc.26054.

Gamma-tocotrienol induces apoptosis and autophagy in prostate cancer cells by increasing intracellular dihydrosphingosine and dihydroceramide

Qing Jiang[*], **Xiayu Rao**, **Choon Young Kim**, **Helene Freiser**, **Qingbo Zhang**, **Ziying Jiang**, and **Guilan Li**
Department of Foods and Nutrition, Interdepartmental Nutrition Program, Purdue University, West Lafayette, IN 47907

Abstract

Although cell-based studies have shown that γ-tocotrienol (γTE) exhibits stronger anticancer activities than other forms of vitamin E including γ-tocopherol (γT), the molecular bases underlying γTE-exerted effects remains to be elucidated. Here we showed that γTE treatment promoted apoptosis, necrosis and autophagy in human prostate PC-3 and LNCaP cancer cells. In search of potential mechanisms of γTE-provoked effects, we found that γTE treatment led to marked increase of intracellular dihydroceramide and dihydrosphingosine, the sphingolipid intermediates in *de novo* sphingolipid synthesis pathway, but had no effects on ceramide or sphingosine. The elevation of these sphingolipids by γTE preceded or coincided with biochemical and morphological signs of cell death and was much more pronounced than that induced by γT, which accompanied with much higher cellular uptake of γTE than γT. The importance of sphingolipid accumulation in γTE-caused fatality was underscored by the observation that dihydrosphingosine and dihydroceramide potently reduced the viability of both prostate cell lines or LNCaP cells, respectively. In addition, myriosin, a specific inhibitor of *de novo* sphingolipid synthesis, counteracted γTE-induced cell death. In agreement with these cell-based studies, γTE inhibited LNCaP xenograft growth by 53% ($P<0.05$), compared with 33% ($P = 0.07$) by γT, in nude mice. These findings provide a molecular basis of γTE-stimulated cancer-cell death and support the notion that elevation of intracellular dihydroceramide and dihydrosphingosine is likely a novel anticancer mechanism.

Keywords

sphingolipids; vitamin E; tocopherol; autophagy; apoptosis

INTRODUCTION

Vitamin E has eight lipophilic antioxidants, i.e., α-, β-, γ- and δ-tocopherol (αT, βT, γT and δT) and α-, β-, γ- and δ-tocotrienol (αTE, βTE, γTE and δTE). Specific forms of vitamin E have been proposed to be promising anticancer agents [1, 2]. For instance, γT has been shown to suppress neoplastic transformation via scavenging reactive nitrogen species [3], and inhibit growth and induce apoptosis of prostate and colon cancer cells by modulation of sphingolipid metabolism [4], down-regulation of cyclins [5] or up-regulation of peroxisome proliferator activated receptor-γ [6]. Recently, γT-rich mixed tocopherols are reported to

[*]To whom all correspondence should be addressed: Qing Jiang, PhD, Department of Foods and Nutrition, 700 W State Street, Stone Hall G1A, Purdue University, West Lafayette, IN 47907. Phone: (765)-494-2483, Fax: (765)-494-0674, qjiang@purdue.edu.

None of the authors has conflict of interest.

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inhibit tumor development in animal models [2, 7–11]. Besides tocopherols, γTE, a γT analog with an unsaturated side chain and abundant in palm oil, has been reported to exhibit potent anticancer effects in various types of cancer cells [12–16]. In cell-based studies, γTE appears to show stronger efficacy than γT in the anti-proliferation and pro-apoptotic activity [14]. Although several biochemical events associated with γTE-induced anticancer actions have been characterized, including its activation of caspase-8 or JNK, induction of endoplasmic reticulum (ER) stress and inhibition of PI3K-mediated AKT phosphorylation [12–17], the molecular bases that trigger these changes as a result of γTE treatment remain ambiguous.

Emerging evidence suggests that modulation of *de novo* synthesis of sphingolipids may play a role in cell fatality and therefore may be a useful strategy in chemoprevention and therapy [18, 19]. We have demonstrated that γT treatment leads to intracellular accumulation of dihydroceramide and dihydrosphingosinge (sphinganine), two key sphingolipid intermediates in the *de novo* synthesis of sphingolipid pathway, in prostate LNCaP cells, and this action appears to contribute to γT-related apoptosis [4]. Fenretinide, a chemotherapeutic agent being tested in clinical trails, has been shown to enhance dihydroceramide in cancer cells [19, 20]. Since γTE was reported to exhibit much more potent anticancer activity than γT in prostate cancer cells [14], we hypothesize that γTE may also modulate sphingolipid metabolism, which may play a role in γTE's pro-death effect on cancer cells. In the present study, we characterized the effect of γTE on cell fatality of human prostate PC-3 and LNCaP cancer cells, investigated whether γTE is capable of modulating intracellular sphingolipids, and examined the anticancer efficacy of γTE on LNCaP-xenograft tumor growth in nude mice. We also compared the relative effectiveness of γTE with that of γT in these activities.

MATERIALS AND METHODS

Materials and reagents

γ-Tocotrienol (>95%) was a gift from Dr. Klaus Kramer from BASF (Germany). γT (> 95%) was purchased from Sigma (St Louis, MO) or Acros Organic (New Jersey). Tissue culture reagents were from GibcoBRL (Rockville, MD). The pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (Z-VAD-fmk) and fatty acid free bovine serume albumin (BSA) were from CalBiochem (San Diego, CA). Tocopherol-stripped corn oil was obtained from Dyets Inc. (Bethlehem, PA). Dihydrosphingosine (sphinganine) was from Avanti Polar Lipids, Inc (Alabaster, AL). Myriocin from *Mycelia Sterilia,* C2-dihydroceramide, dimethyl sulfoxide (DMSO), [3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), mevalonate and all other chemicals were from Sigma (St Louis, MO).

Cell culture and effects of vitamin E forms and sphingolipids on cell viability

Human prostate cancer cell lines, PC-3 and LNCaP cells, were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS). At the time of experiments, cells were seeded in RPMI-1640 with 10% of FBS at a density of $3–4 \times 10^4$ cells/well in 24-well plates. Twenty-four or forty-eight hours later, media were replaced with fresh RPMI containing 1% of FBS and vitamin E forms. Before added to media with 1% FBS, γTE and γT were dissolved in DMSO at 50–100 mM and then diluted to 5 mM in fatty acid-free BSA (5 mg/ml). During preparation, samples were kept cold and exposure to light was avoided.

Dihydrosphingosine and C2-dihyceramide were dissolved in DMSO and were then added to RPMI containing 1% of FBS media. In all experiments, the final concentration of DMSO did not exceed 0.15%. The number of viable cells was quantified by the MTT assays [21].

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Evaluation of apoptosis and necrosis by annexin V and propidium iodide (PI) staining

Both floating and attached cells were collected by brief trypsinization. Cells were stained with Annexin-V-Fluos staining kit from Roche Applied Science and apoptosis was evaluated using Becton Dickinson FACSort (BD Biosciences). Annexin V recognizes the externalization of phosphatidylserine of the plasma membrane, a marker of apoptosis and PI penetrates into plasma membrane of cells that have lost membrane integrity (necrosis).

Isolation of cytosolic fraction

Cells were homogenized in the buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and protease inhibitors [22]. The homogenate was centrifuged at x400g, 4 °C for 10 min, and the supernatant was further centrifuged at x10000g for 10 min. The resultant supernatant was used as the cytosolic fraction. Protein amount was determined by the BCA protein assay kit (Pierce, Rockford, IL).

Western Blot

Cells were lysed in Tris-EDTA, 1%SDS, 1 mM DTT with protease inhibitor cocktails (Sigma) and the resulting solution was heated at 95 °C for 5 min. To measure Akt phosphorylation, cells were collected by scraping. After a brief centrifugation, cell pellets were lysed in Tris-EDTA, 1%SDS, 1 mM DTT with 2 mM Na_3VO_4. 10–25 μg of proteins was loaded on 10–12% pre-cast SDS-PAGE gels (BioRad, Richmont CA), semi-dry transferred onto PVDF membrane (Millipore) and probed by antibodies. Membranes were exposed to chemiluminescent reagent (NEN, Life Science Products) and visualized on a Kodak film.

Lipid extraction

Lipids were extracted as previously described [23, 24]. Briefly, cell pellets were resuspended in chloroform/methanol/1N hydrochloric acid (100:100:1; v/v/v) by tip sonicated for 20 s. After 0.25 volume of 1M sodium chloride was added to achieve phase separation, the lower organic phase was recovered. Ten percent organic phase was used to determine total choline-containing phospholipids (Ch-PL) by an enzymatic colorimetric assay (Wako Chemicals GmbH, Germany). The rest organic phase was divided into two fractions with addition of C14-sphingosine (150 pmol) as the internal standard, and dried under N_2. One fraction was hydrolyzed in 0.5 mL of 1M potassium hydroxide in methanol at 90 °C for 1 h to convert ceramide or dihydroceramide to sphingoid bases. The other lipid fraction was incubated in 0.1M sodium hydroxide in methanol at 37 °C for 1 hr for measurement of free sphingoid bases.

Measurement of sphingolipid intermediates using HPLC with fluorescent detection

Sphingolipid intermediates were derivatized using *o*-phthalaldehyde to form fluorescent derivatives that were separated by HPLC and detected by a Shimadzu RF-10AXL spectrofluorometric detector (Shimadzu, Columbia, MD) with the excitation and emission wavelength at 340 nm and 455 nm, respectively [23, 24].

Cellular uptake of γTE and γT

Cells were incubated with γTE and γT at specified concentrations in RPMI-1640 media with 1% FBS for 6 h. After being washed twice with media, Cells were collected and homogenized by tip sonication. Vitamin E forms were extracted and analyzed by HPLC with electrochemical detection as previously described [25].

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Animal study

The animal use protocol was approved by the animal care committee at Purdue University and strictly followed. LNCaP cells were collected from exponentially growing cultures that were 70–80% confluent. Collected cells were mixed with Matrigel (Becton Dickson Labware) 1:1 by volume. A total volume of 150 μL cell/Matrigel mixture containing 3×10^6 cells was injected subcutaneously into the right flank of 6–7 week old male BALB/c nude mice (purchased from NCI Frederick Cancer Research Center). Twenty-four hours later, mice were randomized into control (Ctrl), γT- or γTE-treated groups. γT and γTE were administrated by gavage at 125 mg/Kg body weight three times a week, using tocopherol-stripped corn oil as the vehicle (0.1 mL). Control animals were given 0.1 mL corn oil. Tumor size ($L\times W^2\times0.5$), food intake and body weight were measured once a week.

RESULTS

γTE treatment induced apoptosis, necrosis and autophagy in human prostate PC-3 and LNCaP cells

γTE treatment led to a time and dose-dependent decrease in cell viability of PC-3 and LNCaP cells, as indicated by the MTT assays, and PC-3 cells appeared to be slightly more sensitive than LNCaP to the treatment (Fig. 1). Examination of cell morphology revealed a time- and dose-dependent cell detachment and vacuole formation in the cytoplasm resultant from γTE incubation, suggesting induction of cell death. Compared with our previous studies on γT [4], γTE showed much more effective anticancer activity because γTE at 20 μM decreased cell viability by 80–90% in both cell lines within 48 hours, whereas γT at 50 μM reduced cell viability by 50–60% after 72–96 h incubation and failed to induce death in PC-3 cells. These observations were also in agreement with a recent study by Yap *et al* [14].

The nature of γTE-induced cell death was first analyzed by propidium iodide (PI) and annexin V double staining using flow cytometry. γTE treatment appeared to cause apoptosis and necrosis in PC-3 cells (Fig. 1C) and LNCaP cells (Fig 1 in supplement). Consistent with induction of apoptosis, γTE treatment led to PPAR cleavage and the release of cytochrome C to the cytosol (Fig. 2). Activation of caspase-9 was indicated by the decrease of procaspase 9 (47 kDa) in PC-3 cells and the increase of the activated caspase 9 (37 or 35 kDa) in LNCaP cells (Fig. 2A). Caspase activation was also supported by the observation that z-VAD-fmk, an irreversible pan-caspase inhibitor, partially prevented γTE-induced activation of caspase 9 (Fig. 2B). However, z-VAD-fmk failed to counteract γTE-caused cell death (Fig. 2C), suggesting potential involvement of different types of death. Richmann *et al* recently reported that tocotrienols induce autophagy in pancreatic stella cells [26]. Our western blotting data showed that γTE treatment led to an increase of membrane bound microtubule-associated protein light chain 3 (LC3II), a marker of autophagy [27], in both PC-3 and LNCaP cells (Fig. 2D). These results therefore indicate that γTE treatment induced autophagy, in addition to apoptosis and necrosis.

γTE did not affect Akt phosphorylation until the late stage of cell death and mevalonate did not reverse γTE-induced effects

In search of the upstream pathway(s) that may be influenced by γTE treatment, we found that γTE had no effect on BCl-2 or BCl-xL expression (unpublished observation). Unlike observations in mouse mammal cells [15, 17], γTE did not activate caspase 8 in the prostate cancer cells (data not shown). A previous study showed that γTE treatment led to suppression of Akt phosphorylation [12]. However, we found that γTE did not affect Akt phosphorylation in LNCaP and PC-3 cells after 16-h treatment when cell death was evident (*comp*. Fig. 2 and Fig. 3), whereas wortmannin (at 1 μM), a specific inhibitor of PI3K, effectively decreased Akt phosphorylation at both 16 and 24 h (Fig. 3). These results

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indicate that inhibition of PI3K signaling was not a causal factor in γTE-induced death. In addition, although γTE is known to inhibit HMG CoA reductase-mediated mevalonate formation in cholesterol biosynthesis pathway [28], co-incubation with mevalonate failed to reverse γTE-induced cell death (data no shown), suggesting that suppression of HMG-CoA does not contribute significantly to the cell fatality.

γTE treatment induced marked accumulation of dihydrosphingosine and dihydroceramide in both PC3 and LNCaP cells, which accompanied with high cellular uptake of this vitamin E form

We have previously shown that γT treatment led to accumulation of intracellular sphingolipid intermediates, which may be responsible for γT-induced apoptosis in LNCaP cells [4]. Here we found that in both LNCaP (Fig. 4A and 4B) and PC-3 cells (Fig. 4C and 4D), γTE caused marked increase of dihydrosphingosine and dihydroceramide, which are sphingolipid intermediates in *de novo* synthesis pathway of sphingolipids. The extent of sphingolipid accumulation induced by γTE was much more pronounced than that by γT (Fig. 4). Importantly, γTE significantly elevated dihydroceramide and dihydrosphingosine at 8 and 16 h after incubation (Fig. 4C and 4D), which preceded and/or coincided with activation of death-related biochemical events such as PARP activation (Fig. 4E). In most experiments, extensive cell detachment, a morphological indication of cell death, was evident at 16–24 h after γTE incubation. On the other hand, γTE treatment had no significant effect on ceramide or sphingosine even after 24 h incubation (data not shown).

To understand potential reason(s) for the much more potent effects of γTE than γT on sphingolipid accumulation and pro-death activity, we compared the relative cellular uptake of these two vitamin E forms. The results showed that cellular accumulation of γTE in PC-3 and LNCaP cells appeared to be much higher than that of γT (Fig. 4F).

Myriosin significantly counteracted γTE-induced death; Dihydrosphingosine and dihydroceramide reduced cell viability in PC-3 and LNCaP cells

To investigate the importance of sphingolipid increase in γTE-induced cell death, we used myriosin, a specific inhibitor of serine palmitoyl CoA transferase, to block the *de novo* synthesis of sphingolipids, and found that myriosin significantly counteracted γTE-induced cell death in LNCaP cells (Fig. 5A). In PC-3 cells, myriosin alone caused significant reduction of cell viability (our unpublished observations), which prevents appropriate evaluation of its effect on γTE-caused cell death.

To further evaluate the role of increased sphingolipid intermediates in mediating cell death, we examined the effect of dihydrosphingosine and dihydroceramide on PC-3 and LNCaP cell proliferation. Treatment with dihydrosphingosine led to rapid and marked cell death in both PC-3 and LNCaP cells, as indicated by MTT assays (Fig. 5B and 5C) and extensive cell detachment via microscopic observations. C2-dihydroceramide also reduced the viability of LNCaP cells but had little effects on PC-3 cells after 24 h treatment (Fig. 5B and 5C). These observations, together with the fact that the elevation of these sphingolipids by γTE took place prior to the death-related molecular changes, support the notion that intracellular increase of dihydroceramide and dihydrosphingosine likely play a significant role in γTE-induced cell death.

γTE significantly suppresses tumor development in nude mice implanted with LNCaP cells, and was more effective than γT in this action

To translate the cell-based studies to an *in vivo* cancer model, we examined the effect of γTE and γT on the growth of LNCaP xenograft in nude mice, and compared their relative potency. Oral gavage of γTE and γT did not have any effects on the body weight (Fig. 6A)

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or food intake (data not shown). Compared with corn oil-fed controls, γTE supplementation significantly inhibited tumor development as indicated by 53% ($P < 0.05$) reduction of tumor volume, while γT reduced ~30% growth (Fig. 6B). The relative efficacy of these two vitamin E forms paralleled that observed in the cell-based studies.

DISCUSSION

We showed that in human prostate cancer cells, γTE treatment led to apoptosis, necrosis and autophagy, and caused marked accumulation of dihydrosphingosine and dihydroceramide, important sphingolipids in *de novo* biosynthesis pathway. On the other hand, γTE did not significantly affect ceramide or sphingosine. The induction of intracellular dihydrosphingosine and dihydroceramide accumulation is likely a key molecular basis responsible for triggering γTE-induced cell death. First, the enhancement of these sphingolipids by γTE preceded or coincided with biochemical and morphological indications of cell death (Fig. 4). Secondly, since dihydroceramide and especially dihydrosphingosine appear to be potent mediators of cell death (Fig. 5), their elevation inside cells likely, at least in part, contributes to γTE-related fatality. Consistent with this notion, the extent of sphingolipid accumulation matches the relative efficacy of cell death induction, as indicated by the higher effectiveness of γTE than γT in both activities. In addition, myriosin, a specific inhibitor of the first reaction in *de novo* synthesis of sphingolipids, counteracted γTE-caused cell death.

Although ceramide and sphingosine have long been recognized as important modulators of apoptosis [18, 19, 29], emerging evidence suggests that sphingolipids in the *de novo* synthesis pathway also play significant roles in determining cell fate. In particular, dihydrosphingosine (sphinganine) has been reported to potently induce apoptosis in colon cancer cells [30] and was implicated in T cell death [31]. We currently showed that dihydrosphingosine rapidly induced death of PC-3 and LNCaP cells (Fig. 5). Interestingly, safingol, the synthetic L-threo-stereoisomer of dihydrosphingosine, has recently been shown to be an inhibitor of PI3K and induce autophagy in different cancer cells [32]. Since the suppressive effect of γTE on PI3K-mediated Akt phosphorylation was observed after the elevation of intracellular sphingolipids in PC-3 cells in the present study (*comp*. Fig. 3 with Fig. 4), it is conceivable that the enhanced dihydrosphingosine (by γTE) may lead to γTE-induced autophagy and suppression of Akt phosphorylation. In addition, C2-dihydroceramide, despite being less potent than dihydrosphingosine, inhibited the growth of neuroblastoma cells [20] and reduced the viability of LNCaP cells (Current study). Consistent with these observations, down-regulation of dihydroceramide desaturase, which results in dihydroceramide accumulation, leads to inhibition of cell growth and cell cycle arrest [20], while over-expression of this enzyme upregulates cyclin D1 and enhances metastatic efficiency in esophageal carcinoma cells [33].

Because of the significant role of dihydrosphingosine and dihydroceramide in promoting cancer cell fatality, compounds that are capable of modulation of *de novo* synthesis of sphingolipid may have valuable chemo-preventive or therapeutic property. In this regard, γT is the first natural compound identified to induce apoptosis by enhancing intracellular dihydrosphingolsine and dihydroceramide [4]. Since then, more chemoprevention or chemotherapeutic agents, which bear different structures and specificities, have been reported to modulate intracellular levels of these sphingolipids. Fenretinide, a synthetic analog of *all-trans*-retinoic acid and chemotherapeutic agent that induces cancer cell death or G1/S arrest, enhances intracellular dihydroceramide [19] by inhibition of dehydroceramide desaturase activity [20]. Recently, resveratrol is reported to increase dihydroceramide in gastric cancer cells [34]. Celecoxib, a selective cyclooxygenase-2 inhibitor and known to cause cancer cell death via cyclooxygenase-independent mechanisms [35], is shown to

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increase intracellular accumulation of dihydroceramide and dihydrosphingosine by inhibiting the activity of dihydroceramide desaturase and stimulating *de novo* synthesis of sphingolipid pathway [36]. Our current study demonstrates that γTE is much more potent than γT in induction of dihydrosphingosine and dihydroceramide. Treatment with γTE and γT also led to accumulation of these sphingolipids in colon cancer cells (Rao and Jiang, unpublished data). Based on these results, we propose that elevation of intracellular dihydrosphingosine and dihydroceramide may be a general pathway of induction of cancer cell death, although the exact mechanisms leading to this effect remains to be elucidated. Further studies should be carried out to determine whether vitamin E forms affect the expression or activity of dihydroceramide desaturase, whether they have impacts on the *de novo synthesis* of sphingolipids, and whether they have differential effects on different dihydroceramide or ceramide species using sphingolipodomics [19] because individual ceramide appears to have distinct bioactivities [37].

The observation that PC-3 and LNCaP cells appeared to accumulate much more γTE than γT may, in large part, explains the stronger pro-death and induction of sphingolipid accumulation by γTE. However, in contrast to its higher cellular uptake, γTE appears to be more rapidly metabolized than γT *in vivo* [25]. It is therefore necessary to examine the relative effectiveness of these vitamin E forms in animal models. To this end, we employed LNCaP- rather than PC-3-xenograft nude mouse model because both γTE and γT treatment caused LNCaP cell death while γT was much less effective toward PC-3 cells[4]. Our results indicate that γTE inhibited prostate tumor growth more effectively than γT in the xenograft model, which is consistent with the cell-based observations. The paradox between rapid *in vivo* metabolism and high cellular uptake of γTE may suggest that γTE tends to be accumulated by tumor cells or tissues, which warrants further investigation. The mechanisms underlying differential cellular uptake between γTE and γT need to be further explored.

Although many cell-based studies have reported the anti-cancer activity of γTE, research on testing its efficacy in animal models is relatively limited and only emerged recently. In addition to our current study, Park *et al* showed that γTE moderately suppressed the growth of implanted-mammary tumor in female BALB/c mice [17]. γTE appears to synergize with gemcitabine to inhibit pancreatic cancer growth in athymic nu/nu mice [38]. These studies demonstrate that cell-based anticancer effects of γTE can be translated into animals. However, xenograft models do not always provide good clinical prediction of drug effectiveness in human due to their lack of imitation of the complexity of human cancers. In this regard, transgenic mice bearing genetic defects associated with human cancer development ought to be used to further examine chemoprevention and therapeutic potential of vitamin E forms. Interestingly, mixed tocotrienols are recently shown to inhibit prostate carcinogenesis in the TRAMP mice [39], which is in agreement with our study in LNCaP-xenograft nude mice. It is also noteworthy that in addition to the pro-death effects, γTE and γT as well as their metabolites have been demonstrated to have anti-inflammatory activities by inhibiting cyclooxygenase- and 5-lipoxygnease-catalyzed generation of eicosanoids[40–42], which are well recognized pro-carcinogenic agents [43]. Therefore, future studies with preclinical models including transgenic mice should examine various anticancer mechanisms exerted by γTE and other vitamin E forms including pro-death, modulation of sphingolipids and anti-inflammatory actions.

Acknowledgments

This study was in part supported by grants from NIH R01AT001821 (QJ) and R21CA133651 (QJ).

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ABBREVIATIONS

α-T,β-T, γ-T, or δ-T	α, β, γ, or δ-tocopherol
αTE, βTE, γTE, δTE	α, β, γ, δ-tocotrienol
PI3K	phosphoinositide 3-kinase
PI	propidium iodide
LC-3	microtubule-associated protein light chain 3

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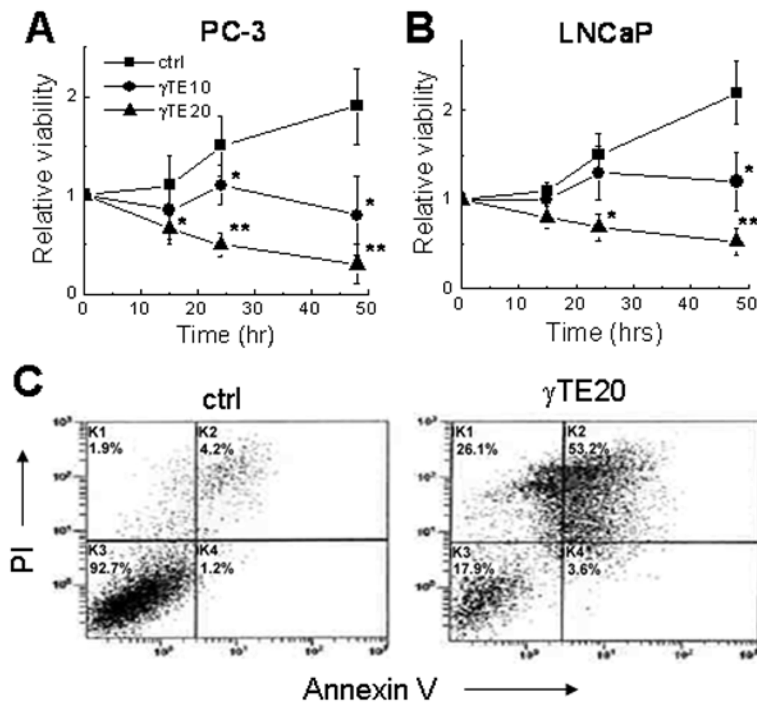

Figure 1. Effects of γTE on the viability of PC-3 (A) and LNCaP (B) cells and evidence for γTE-induced apoptosis and necrosis in PC-3 cells (C)

Cells were treated by γTE (indicated in μM) or DMSO (controls) and the effect on cell viability was evaluated at indicated times using MTT assays. Data are the averages of two to three independent experiments. *P<0.05 and **P<0.01 indicate a significant difference between treated and control cells. Flow cytometry was performed to evaluate apoptosis (annexin V+ and annexin V+/PI+) and necrosis (PI+) in PC-3 cells after 24-h treatment with DMSO (ctrl) or γTE at 20 μM.

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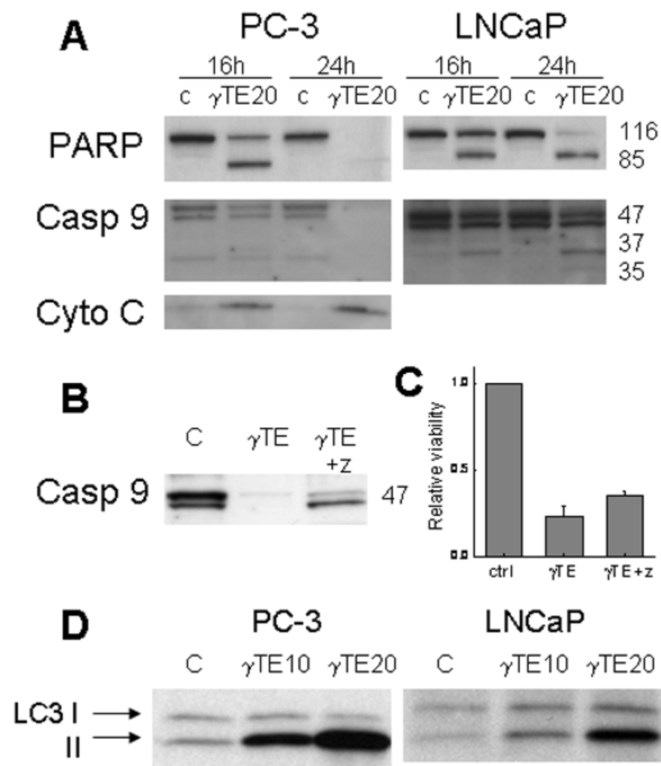

Figure 2. Biochemical events associated with cell death induced by γTE treatment

Panel A - Compared with DMSO control (c), γTE (20 μM) led to PARP cleavage, caspase-9 (casp 9) activation and cytochrome C (Cyto C) release to the cytosol. **Panel B** - Z-VAD-fmk (z) (30 μM) inhibited γTE-caused caspase-9 activation in PC-3 cells. **Panel C** - Z-VAD-fmk (z) did not counteract γTE-induced death as indicated in MTT assays. **Panel D** -γTE (10 or 20 μM) treatment resulted in elevation of LC-3II, a marker of autophagy.

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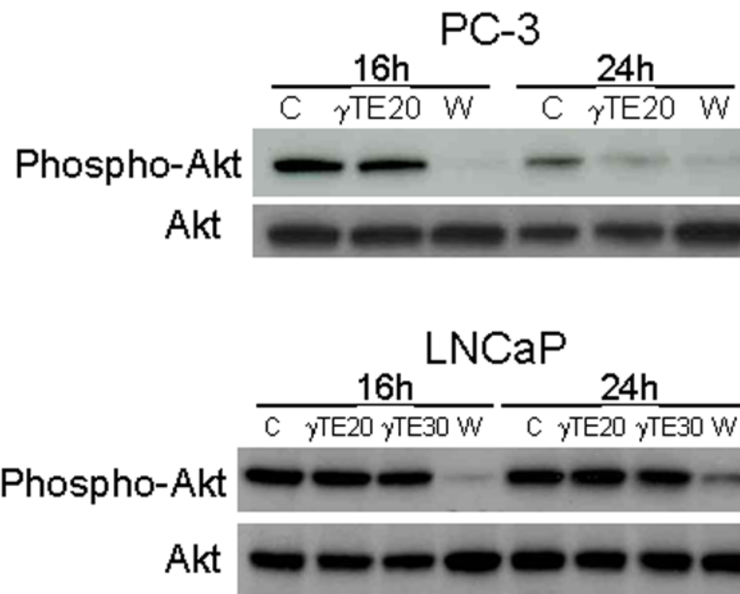

Figure 3. The effect of γTE on PI3K-mediated Akt phosphorylation in PC-3 and LNCaP cells
Cells were treated with γTE at 20 (γTE20) or 30 μM (γTE30) or wortmannin (W, 1 μM) for indicated times. Western blotting was performed to evaluate the effect on Akt phosphorylation.

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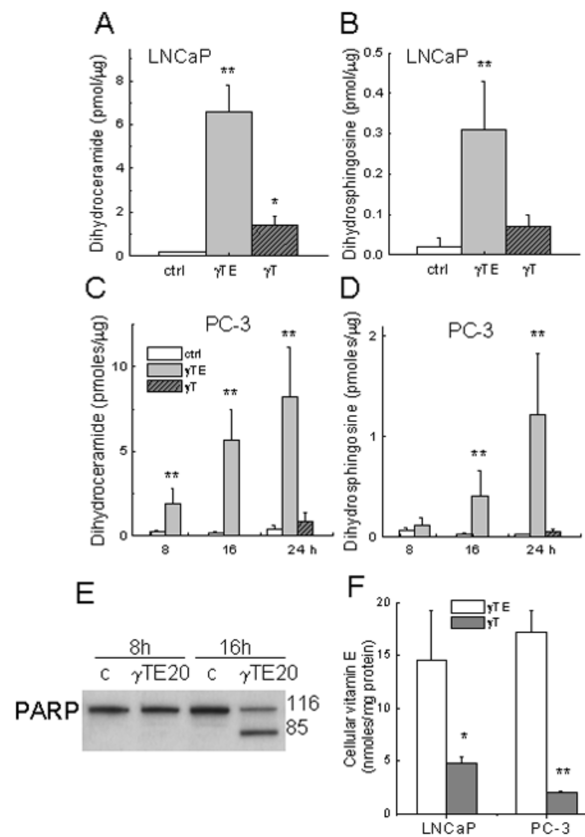

Figure 4.

Panels A and B - γTE (20 μM) or γT (50 μM) induced accumulation of dihydroceramide and dihydrosphingosine in LNCaP cells after 24-h incubation. **Panels C and D** - Effects on sphingolipids by γTE (20 μM) at indicated times or γT (50 μM, 24 h) in PC-3 cells. In Panels A–D, $^{**}P<0.01$ and $^{*}P<0.05$ indicate difference between γTE-treated and control cells. **Panel E –** Effects of γTE (20 μM) on PARP cleavage at indicated times in PC-3 cells. **Panel F** – Cells were incubated with γTE (10 μM) or γT (50 μM) in RPMI-1640 with 1% FBS for 6 h. Vitamin E forms extracted from collected cells were analyzed by HPLC with electrochemical detection. $^{**}P<0.01$ and $^{*}P<0.05$ indicate difference between γTE- and γT-treated cells.

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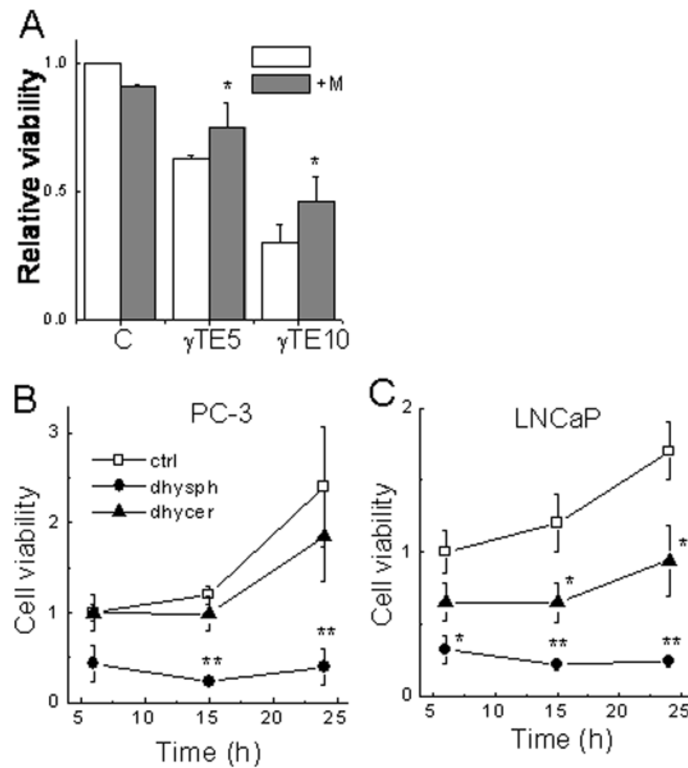

Figure 5.

Panel A - Myriocin (6 μM) significantly protected LNCaP cells from γTE- induced death as indicated by MTT assays. *P<0.05, the difference between cells with myriocin (+M, solid bar) and without myriocin (open bar). **Panel B and C** - Effects of dihydrosphingosine and dihydroceramide on the viability of LNCaP and PC-3 cells. Cells were treated with dihydrosphingosine (dhysph, 25 μM) and dihydroceramide (dhycer, 25 μM) for indicated times and cell viability was analyzed by the MTT assays. **P<0.01 and *P<0.05, the difference between sphingolipid treatment and controls.

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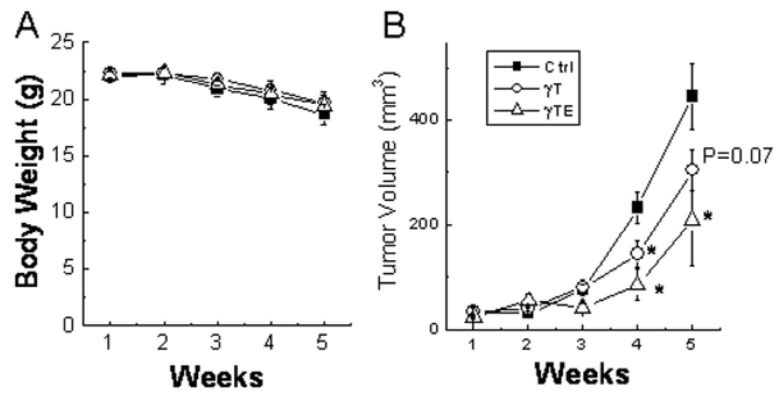

Figure 6. γTE and γT inhibited LNCaP-xenograft growth in nude mice

Male BALB/c nude mice were s.c. injected with LNCaP cells. Animals were gavaged with γT, γTE (at 125 mg/Kg bw in 0.1 mL corn oil) or corn oil alone (Ctrl) three times a week. Body weights were measured once a week (Panel A). Tumor volumes were assessed and expressed as mean ± SE (n = 7–8) (Panel B). *P<0.05 indicates significant difference between treated and control groups.