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Induction of DNA Damage and Caspase-Independent Programmed Cell Death by Vitamin E

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Vitamin E comprises 8 functionally unique isoforms and may be a suitable candidate for the adjuvant treatment of prostate cancer. In this study, we examined the ability of 2 vitamin E isoforms [\(\alpha\)-tocotrienol (\(\gamma\)-TT) and \(\delta\)-tocotrienol (\(\delta\)-TT)] and 4 synthetic derivatives [\(\gamma\)- and \(\delta\)-tocotrienol succinate (\(\gamma\)-TS, \(\delta\)-TS), \(\alpha\)-tocopheryl polyethylene glycol succinate (TPGS), and \(\alpha\)-tocopheryl polyethylene glycol ether (TPGS-e)] of vitamin E to induce cell death in AR− (DU145 and PC-3) and AR+ (LNCaP) prostate cancer cell lines. Our results show that \(\delta\)-TT and TPGS-e are the most effective isoform and synthetic derivative, respectively, of all compounds examined. Overall, the results of our study suggest that isoforms and synthetic derivatives of vitamin E have the potency to trigger both caspase-dependent and -independent DNA damage and dominant caspase-independent programmed cell death. The capacity of vitamin E to trigger caspase-independent programmed cell death suggests that it may be useful in the chemotherapy of prostate cancer since it may prevent the tumor resistance commonly associated with the use of classical chemotherapeutic agents that trigger caspase-dependent programmed cell death.

INTRODUCTION

Prostate cancer is the second most common form of cancer with respect to mortality and the cancer with highest incidence in males (1). Several studies have shown that vitamin E has important anticancer activity and may therefore be useful in the treatment of prostate cancer (2–11).

Vitamin E exists in nature as a group of 8 isoforms: \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-tocopherols (\(\alpha\)-TOC, \(\beta\)-TOC, \(\gamma\)-TOC, and \(\delta\)-TOC, respectively) and \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-tocotrienols (\(\alpha\)-TT, \(\beta\)-TT, \(\gamma\)-TT, and \(\delta\)-TT, respectively) (11–14). Evidence in the literature suggests that the anticancer activity of vitamin E isoforms is associated with their ability to induce apoptosis (15–18). The order of apoptotic efficiency of vitamin E tocopherols is considered to be \(\delta\)-TOC > \(\gamma\)-TOC > \(\beta\)-TOC > \(\alpha\)-TOC (7,19). Despite the fact that the serum concentration of the tocotrienols is much lower than that of the tocopherols (20), the former display greater antitumor activity than the latter (9,16–19,21) In general, the apoptotic potency of the tocotrienols is considered to be \(\delta\)-TT > \(\gamma\)-TT > \(\beta\)-TT > \(\alpha\)-TT (19,22), and there is evidence in the literature that suggests the most potent tocotrienols \(\gamma\)-TT and \(\delta\)-TT induce caspase-dependent programmed cell death (CD-PCD) (15–17,23).
In addition to CD-PCD, other forms of programmed cell death (PCD) also exist in the cell. Evidence is beginning to accumulate supporting the existence of caspase-independent programmed cell death (CI-PCD) [reviewed by Constantinou et al. (24)]. Even though the exact signaling pathways triggered during the induction of CI-PCD have not yet been discovered, these pathways should be unraveled as they are not only induced by chemotherapeutic drugs in clinical use (e.g., doxorubicin, camptothecin, paclitaxel, cladribine, and cisplatin) but also by several natural agents with anticancer properties (e.g., selenite, vitamin D, lipoic acid, resveratrol) (24). Evidence in the literature suggests that vitamin E isoforms may also be involved in the induction of CI-PCD (25,26). Nevertheless, the details of these mechanisms have not been thoroughly investigated.

Research in the past few years has focused on structural variations on \( \alpha \)-TOC with the aim to enhance the proapoptotic potency of these agents (27,28). Two examples of synthetic derivatives developed and tested are \( \alpha \)-tocopherol succinate (\( \alpha \)-TOS) and \( \alpha \)-tocopherol ether-linked acetic acid (\( \alpha \)-TEA). Previous studies have shown that the conversion of \( \alpha \)-TOC to \( \alpha \)-TOS and \( \alpha \)-TEA greatly improves its anticancer action in tumorigenic cell lines and animal models, thereby supporting the need for further evaluation of synthetic derivatives in in vitro and in vivo systems (29–36).

The results of a study recently completed in our laboratory have shown that the tocotrienols \( \gamma \)–TT and \( \delta \)–TT and the synthetic derivatives \( \gamma \)–TS and \( \delta \)–TS induce dominant CI-PCD in AR− (DU145, PC3) and AR+ (LNCaP) prostate cancer cell lines (19). In order to investigate this further in the current study, we examined the molecular events activated following treatment of the aforementioned AR− and AR+ cell lines with the promising compounds \( \gamma \)–TT, \( \delta \)–TT, \( \gamma \)–TS, and \( \delta \)–TS in addition to the 2 synthetic derivatives \( \alpha \)-tocopheryl polyethylene glycol succinate (TPGS) and \( \alpha \)-tocopheryl polyethylene glycol ether (TPGS-e). TPGS is a well-known compound, but TPGS-e is a novel compound that is examined for the first time in the current article (refer to Fig. 1 for structure of the compound). The synthetic derivatives were examined in addition to the isoforms in order to investigate whether 1) structural modifications of the isoforms improve their proapoptotic potency, and 2) different compounds regulate the induction of different pathways of PCD. Furthermore, the isoforms and synthetic derivatives of vitamin E (VitE-ISDs) were examined in 3 different cell lines in order to explore the role that the cellular microenvironment may have on the regulation of different pathways of cell death. Overall, the results of our study have shown that all VitE-ISDs examined \( \gamma \)-TT and TPGS-e were the most effective with regard to their ability to induce cell death. Our results have also shown that \( \gamma \)-TS and \( \delta \)-TT trigger caspase-dependent DNA damage in the AR+ cell line LNCaP, whereas all other VitE-ISDs trigger caspase-independent DNA damage in both AR+ and AR- cell lines. Nevertheless, all VitE-ISDs (\( \gamma \)-TS, \( \delta \)-TS, TPGS, and TPGS-e) induce dominant caspase-independent programmed cell death (CI-PCD) in all cell lines examined.

**MATERIALS AND METHODS**

**Reagents**

DMEM, fetal bovine serum, antibiotic/antimycotic and trypsin used in cell culture were purchased from Gibco, Invitrogen (Carlsbad, CA). The caspase inhibitor benzylloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z.vad.fmk) was purchased from Calbiochem (Darmstadt, Germany). The caspase-3/CPP32

**FIG. 1.** The structures of vitamin E isoforms \( \gamma \)-tocotrienol (\( \gamma \)-TT) and \( \delta \)-tocotrienol (\( \delta \)-TT) and vitamin E synthetic derivatives \( \gamma \)-tocotrienol succinate (\( \gamma \)-TS), \( \delta \)-tocotrienol succinate (\( \delta \)-TS), \( \alpha \)-tocopheryl polyethylene glycol succinate (TPGS), and \( \alpha \)-tocopheryl polyethylene glycol ether (TPGS-e).
Fluorogenic substrate was obtained from Kamiya (Seattle, WA). The caspase-3, caspase-8, caspase-9, Bcl-2, AIF, and PARP antibodies were all purchased from Cell Signaling Technology (Danvers, MA). α-Tubulin antibody was purchased from Sigma (St. Louis, MO). TPGS was purchased from Eastman Chemical Company (Kingsport, TN). All other reagents were purchased from Sigma (St. Louis, MO).

**Vitamin E Natural Isoforms and Synthetic Derivatives**

**Isolation of d-γ-Tocotrienol (γ-TT)**

γ-TT was isolated from approximately 50% total tocol-containing concentrate of palm oil (Tocomin-50R) obtained from Carotech, Inc. (Ipoh, Malaysia). This concentrate contained approximately 20% γ-TT, 11% α-TT, and a total of 19% other tocopherols and tocotrienols. The remainder of the concentrate was largely comprised of triglycerides, fatty acids, fatty alcohols, carotenoids, and sterols. Approximately 60 g samples of Tocomin-50 were chromatographed on open columns containing 1.5 kg of silica gel. The chromatography was followed using thin-layer silica gel chromatography (read using UV fluorescence and p-anisaldehyde spray reagent) and proton NMR spectroscopy of concentrated fractions. Elution was with a gradient from pure hexanes to 12% acetone in hexanes using approximately 5–8 liter increments containing, respectively, 0.5, 1.0, 2.0, 4.0, 7.0, and 12% acetone in hexanes. The tocotrienol-containing fraction (7% acetone and above) was stripped of solvent and rechromatographed on silica gel in the same way to give 2 fractions that contained approximately 75% of γ-TT. In order to remove non-tocol impurities, which co-eluted with the 2 desired tocotrienols, the enriched fractions were acetylated. The fractions enriched in γ-TT were each stripped of solvent on the rotovap, redissolved in 20 ml each of pyridine, and treated with 10 ml of acetic anhydride. After stirring overnight at room temperature, the 2 acetylation mixtures were quenched by the addition of a few ml of water followed by stirring for 2 h. The reaction mixtures were then poured into 500 ml of water, extracted with ethyl acetate, and the ethyl acetate layers washed with 5% brine, dried over sodium sulfate, and stripped on the rotovap, diluted with water, neutralized with dilute HCl, and extracted with ethyl acetate. The organic phase was washed with brine, dried over sodium sulfate, and stripped on the rotovap. The resulting tocotrienol was flash-chromatographed on silica gel (acetone-hexane gradient elution) to give about 3.5 g of >95% pure (NMR, HPLC) γ-TT as pale yellow viscous oils.

The compound had IR and NMR spectra in agreement with literature values.

**Isolation of d-δ-Tocotrienol (δ-TT)**

Multigram quantities of δ-TT were obtained in 95% purity using the methods described above for α-TT and γ-TT, the principle difference being that the starting feedstock was a δ-tocotrienol-rich fraction of annatto oil (Deltagold® 50) obtained from American River Nutrition, Inc. (Hadley, MA).

**Synthesis γ- and δ-Tocotrienyl Succinate (γ-TS, δ-TS)**

The tocotrienyl hydrogen succinates are well-known compounds, described previously (37–39). For our research, the 2 commercially unavailable tocotrienol succinates γ-TS and δ-TS were all prepared as follows: A solution of 0.50 g (0.00126 mole) of γ-TT or δ-TT in 10 ml of anhydrous pyridine was stirred at 20°C under nitrogen atmosphere. A catalytic quantity (approximately 50 mg) of 4-dimethylaminopyridine and 1 g (0.010 mole) of succinic anhydride was added. The reaction mixture was stirred for 20 h, at which time TLC analysis indicated consumption of the starting tocotrienol and formation of a single polar product. There was added to the mixture about 1 g of water and stirring was continued for 2 h to ensure hydrolysis of the excess succinic anhydride employed. The mixture was then poured into 250 ml of water and extracted with ethyl acetate. The extract was washed with 5%aq. HCl (to remove pyridine) and with brine. The solution was dried over anhydrous sodium sulfate and stripped of solvent on the rotovap to give a crude product as pale yellow viscous syrup, completely homogeneous by TLC and HPLC. Final traces of solvent were removed by storing the compound on a high-vacuum line at 22°C for 7 days. All succinates had the expected NMR and IR spectra.

**Synthesis of d-α-Tocopheryl Poly(Ethylene Glycol) Ether (TPGS-e)**

A resealable Carius high-pressure tube was argon-purged and charged with 1 g of d-α-tocopherol (0.0023 mole) and 0.10 g of finely powdered sodium hydroxide. The tube was cooled to −30°C under argon, and 3.00 g (3.4 ml, 0.068 mole) of ethylene oxide was condensed into the tube. A magnetic stir bar was added and the tube was sealed. The sealed tube was then heated in a 150°C oil bath with stirring for 7.0 h. The tube was cooled to −30°C, opened, and the dark viscous oily product diluted with methanol and removed. The solution was stripped of methanol and chromatographed on a column of silica gel (elution solvent was a gradient of 100% chloroform to 5% methanol in chloroform) to give 2.58 g of yellow product that solidified at room temperature. NMR analysis confirmed the structure of the product and indicated that the PEG chain had 18–20 repeat units.

**Cell Culture**

The human prostate cancer cell lines DU-145, PC-3, and LNCaP were obtained from the American Type Culture
Collection (ATCC; Manassas, VA). DU145 (AR-), PC3 (AR-), and LNCaP (AR+) cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. The cells were maintained at 37°C and passaged 2–3 times per week.

Crystal Violet Assay
A total of 1 × 10⁴ DU-145, PC3 or LNCaP cells were seeded per well of a 96-well plate and incubated for 24–72 h. At the end of the incubation period, the growth medium was removed and replaced with fresh DMEM or DMEM supplemented with the various vitamin E compounds and synthetic derivatives (5, 10, 20, or 40 μM) (and where stated in the presence or 20 μM z.vad.fmk), and the plates were incubated for the time periods described in the figure legends. At the end of each incubation period, the medium was removed and 100 μl of 10% formalin was added in each well for 5 min. Subsequently, formalin was removed, and the wells were washed with PBS and incubated with 100 μl of 0.2% crystal violet for 10 min. The wells were then washed with distilled water several times to ensure removal of the dye, and the plates were allowed to dry at room temperature. After drying, 100 μl of acetic acid was added per well, and the plates were incubated at room temperature for 5 min. At the end of the incubation period, 100 μl of acetic acid were added again to each well, and the plates were immediately read on a microplate reader at 620 nm. The absorbance obtained in untreated control cells for each of the 3 cell lines was considered as 100% viability.

Caspase-3 Activity Assay
Caspase-3 activity assays were performed using the Kemiya caspase-3 fluorogenic substrate (Kamiya, Seattle, WA). The cells in 10 mm² plates were treated with the vitamin E natural compounds or synthetic derivatives as described in the figure legends in the presence or absence of the caspase inhibitor z.vad.fmk (Calbiochem, Darmstadt, Germany). At the end of the incubation period, the cells were washed with PBS, the PBS was removed, and the cells were lysed with 150 μl of cell lysis buffer (5 mM EDTA, 5 mM Tris-Hcl, 0.5% NP40, pH 7.4) per plate. The plates were placed on ice and cells were allowed to lyse for 10 min. The cells were mixed in well with a pipette to ensure lysis. The lysate was transferred into a fresh Eppendorf, leaving the pellet behind. The lysates were stored at −70°C until needed for the performance of the assay. When needed, the samples were thawed and used in a Bradford assay to determine protein concentration. Subsequently, 40–80 μg of each sample was added per well of a 96-well plate. The assay buffer mix was then prepared by adding 1 ml caspase assay fluorometric buffer (2.4 g HEPES, 20 g sucrose, 0.2 g CHAPS, dissolved in 200 ml water and pH 7.4 with NaOH) per 1 μl caspase 3 substrate (AC-DEVD-AFC, used at a final concentration of 2.5 μM) per 10 μl of 1M DTT (final concentration 10 mM). Two hundred μl of assay buffer was then added per sample in a well, and the plate was covered in foil and incubated at 37°C for 1–3 h. At the end of the time points, the plate was read on plate reader (excitation 400 nm, emission 505, slit width 15).

Western Blot Analysis
For determining protein levels, cells were seeded at a concentration of 1 × 10⁵ cells per well of a 10 mm plate. Following incubation, cells were washed in PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein extracts were collected by centrifugation at 10,000 rpm for 10 min at 4°C and maintained at −80°C. Protein levels in each sample were measured by using the Bradford method. Extracts were mixed with SDS sample buffer containing mercaptoethanol, boiled for 3 min, and subjected to SDS-PAGE. To study the changes in different proteins, 70–100 mg of protein were loaded in each lane of a polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane and blocked with 5% milk in TBST buffer for 1 h. Membranes were incubated with the appropriate primary antibodies (1:1000 dilutions in 5% BSA) at 4°C overnight, washed with TBST buffer, and then incubated with secondary antibody (1:2000 dilution in 5% milk) for 1 h at room temperature. α-Tubulin levels were also measured as loading control. Blots were developed using a chemiluminescence kit (Santa Cruz, CA).

Comet Assay
For determining oxidative DNA damage, cells were seeded at a concentration of 1 × 10⁵ cells per well of a 6-well plate and allowed to adhere for 48 h. Cells were then treated with the compounds in question for 24 h. Cells were also treated with 100 μM hydrogen peroxide, which induces oxidative DNA damage for 30 min as a positive control. The remainder of the experiment was carried out under dimmed light to prevent DNA damage from ultraviolet light.

Cells were harvested by trypsinization and centrifuged at 2,000 rpm for 5 min in media containing 10% FBS. Cells were washed and resuspended in 500 μl ice cold PBS (calcium and magnesium free to inhibit endonuclease activity). Using 50 μl of cells suspension, cells were mixed with low melting point agarose (molten and at 37°C) in a ratio of 1:10, applied to a CometSlide (Trevigen, Inc.) and allowed to set for 30 min at 4°C. Slides were then electrophoresed for 30 min in alkaline solution (Tris 300 mM, NaOH, pH > 13) at room temperature for 40 min. Slides were then stained with SYBR Green I (Invitrogen, Carlsbad, CA), and data was visualized by epifluorescence microscopy (excitation/emission: 494 nm/521 nm). Cells were given a qualitative score between 0 and 4 based on the overall size of their tail. DNA damage score is calculated based on the
In the current study, γ-TT and δ-TT were isolated and the synthetic derivatives γ-TS, δ-TS, TPGS, and TPGS-e were synthesized as described in the Materials and Methods (Fig. 1). The VitE-ISDs were incubated in the AR− cell lines DU145 and PC3 and the AR+ cell line LNCaP. At the end of the incubation periods, we examined the ability of the 6 VitE-ISDs to induce cell death (via the performance of crystal violet assays) and to activate the effector caspase-3.

The results of our study showed that, as previously reported, (24) all VitE-ISDs examined induce cell death in AR− and AR+ cell lines (Fig. 2). Nevertheless, δ-TT and TPGS-e were the most effective isoform and synthetic derivative, respectively, with regard to their ability to induce cell death (Fig. 2B and 2F). γ-TT, δ-TT, δ-TS, and TPGS induced caspase-3 activity solely in the AR+ LNCaP cells (Fig. 3C). The mean caspase-3 activities induced were as follows: 48 h—γ-TT: 55%, δ-TT: 75%, δ-TS: 81%; 24 h—TPGS: 45% (Fig. 3C). TPGS-e induced the highest caspase-3 activity of all compounds examined in the LNCaP cells (48 h—10 μM: 247%, 20 μM: 751%) (Fig. 3C), whereas lower caspase activities were also visible in PC3 (48 h—10 μM: 23%, 20 μM 342%) (Fig. 3B) and DU145 (24 h—20 μM: 77%) cells (Fig. 3A). Even though in LNCaP cells the caspase activity induced by δ-TS (81%) was similar to that of δ-TT (75%), and the activity induced by γ-TS (18%) was lower than that of γ-TT (55%) (Fig. 3C). Generally, induction of caspase-3 activity by most compounds (γ-TT, δ-TT, δ-TS, TPGS) was only visible in LNCaP cells, suggesting a greater potency of the AR+ cell line compared to the AR− cell lines to induce caspase-mediated signaling pathways of PCD. On the other hand, TPGS-e was the most effective inducer of caspase-3 activity as it induced caspase-3 activity in all cell lines tested, being the most effective in LNCaP cells (Fig. 3).

Induction of Dominant CI-PCD by VitE-ISDs in AR− and AR+ Cell Lines

The lack of activation of caspase-3 by the vitamin E isoforms γ-TT and δ-TT and the synthetic derivatives γ-TS and TPGS in AR− cell lines led us to further examine the dependency of cell death induced by VitE-ISDs on caspase activation. To investigate this, the cells were incubated with the different VitE-ISDs in the presence or absence of the caspase inhibitor z.vad.fmk (Fig. 2). The activity of the caspase inhibitor was confirmed when the same preparation of z.vad.fmk used in the crystal violet assays (Fig. 2) was shown to inhibit the activity (Fig. 4A) and cleavage (Fig. 4C) of caspase-3 [and to prevent the cleavage of PARP induced by the DNA damaging agent etoposide (Fig. 4B)]. The results of these experiments showed that the cell death induced by the VitE-ISDs examined could not be prevented by the caspase inhibitor z.vad.fmk (Fig. 2). This observation was true even when caspase-3 activity was highly induced in LNCaP cells treated with TPGS-e (compare Fig. 3C to Fig. 2F). The results of these experiments, therefore, suggest that VitE-ISDs induce cell death mainly via activation of caspase-independent enzymes, which induce dominant CI-PCD in both AR− and AR+ prostate cancer cell lines.

Investigation of Induction of Caspase-3 Activity by the DNA Damaging Agent Etoposide in AR− and AR+ Cell Lines

The observed activation of caspase-3 by most VitE-ISDs (γ-TT, δ-TT, δ-TS, TPGS) in LNCaP (Fig. 3C) but not in DU145 and PC3 cells (Fig. 3A and 3B) led to the hypothesis that the cellular microenvironment may determine whether CD-PCD or CI-PCD are induced under specific conditions. To investigate this further, we examined the induction of caspase-3 activity by the DNA damaging agent etoposide in the cell lines DU145, PC3, and LNCaP. Etoposide induced caspase-3 activity in DU145 (Fig. 4A) and LNCaP (Fig. 4C) cells but not in PC3 cells (Fig. 4B). These results were also confirmed by the performance of Western blots to detect the cleavage of caspase-3 (Fig. 4B). Consistently, PARP cleavage was highly induced by etoposide in DU145 and LNCaP cells, but only a very minor cleavage was visible in PC3 cells (Fig. 4C). The latter observation suggests that DU145 and LNCaP cells retain the capacity to activate caspase-3 and induce the cleavage of PARP, but caspase activation and therefore PARP cleavage is greatly inhibited in PC3 cells (Fig. 4Aii, Fig. 4B, and 4C). The results therefore suggest that the molecular pathways being induced by VitE-ISDs depend largely on the cellular microenvironment and the structure of the isoform or synthetic derivative used.

Caspase-Dependent and -Independent Cleavage of PARP by VitE-ISDs

In order to further examine the induction of CI-PCD in our system, the 3 cell lines were treated with the test compounds
FIG. 2. DU145, PC3, and LNCaP cells were incubated in 96-well plates for 24 h. The growth medium was removed and replaced with fresh DMEM or DMEM supplemented with 5, 10, 20, or 40 µM of (A) γ-tocotrienol (γ-TT), (B) δ-tocotrienol (δ-TT), (C) γ-tocotrienyl succinate (γ-TS), (D) δ-tocotrienyl succinate (δ-TS), (E) α-tocopheryl polyethylene glycol succinate (TPGS), or (F) α-tocopheryl polyethylene glycol ether (TPGS-e), in the presence or absence of 20 µM z.vad.fmk and the plates were incubated for 48 h. At the end of the incubation period, the cell viability was measured by the performance of a crystal violet assay as described in the Materials and Methods. The absorbance obtained in untreated control cells for each of the 3 cell lines was considered as 100% viability. The results are representative of 3 different experiments and represent the mean ± SEM of 3 different replicates. ∗P value < 0.05, ∗∗P value < 0.01, ∗∗∗P value < 0.001.

for up to 72 h. Cell extracts were prepared and used in Western blotting for the detection of full-length and cleaved PARP. The results of these experiments showed that no changes were observed during 24–48 h of incubation (data not shown). However, after 72 h of incubation with 20 µM γ-TT, δ-TT, γ-TS, or δ-TS, there was generally a decrease in the levels of the full-length 116 kDa PARP without the appearance of the cleaved 89 kDa product (40), with the exception of δ-TT and δ-TS in LNCaP cells where the cleavage product was present (Fig. 5). This observation was consistent with the induction of low levels of caspase-3 activity under these conditions (Fig. 3). As expected, etoposide caused the classic appearance of the 89 kDa
FIG. 3. (A) DU145, (B) PC3, and (C) LNCaP cells were incubated in 96-well plates for 24 h. The growth medium was removed and replaced with fresh DMEM or DMEM supplemented with 20 µM γ-tocotrienol (γ-TT), δ-tocotrienol (δ-TT), γ-tocotrienyl succinate (γ-TS), δ-tocotrienyl succinate (δ-TS), α-tocopheryl polyethylene glycol succinate (TPGS), and 10 µM or 20 µM α-tocopheryl polyethylene glycol ether (TPGS-e), and the plates were incubated for 48 h. At the end of each incubation period, caspase-3 activity assays were performed as described in the Materials and Methods. The results represent the mean ± SEM of 3 different replicates and are representative of 3 different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001, ****P value < 0.0001.

In order to investigate whether the induction of caspase-3 by TPGS-e was associated with cleavage and activation of caspases-8 and -9, we also performed Western blotting for these enzymes. The results of our study showed that TPGS-e was capable of inducing the cleavage of caspase-8 and caspase-9 in addition to the cleavage of caspase-3 (Fig. 6B). The results of this experiment were consistent with the induction of caspase-3 activity (Fig. 3C). Interestingly, the activation of caspases could be prevented by the caspase inhibitor z.vad.fmk, and no caspase activity was observed by TPGS (Fig. 6B) consistent with the inability of the latter to induce caspase activity (Fig. 3C).

In order to investigate further the induction of CD-PCD and CI-PCD by the test compounds, we examined their ability to modify the levels or integrity of Bcl-2, Bax, and AIF, which have previously been shown to be involved in the induction of CI-PCD (24). The experiments have shown that differences in the endogenous levels of Bcl2 exist between the 3 cell lines as the levels of Bcl-2 and Bax in PC3 and LNCaP cells were higher than those in DU145 cells where these proteins were almost undetectable (Fig. 7). Nevertheless, the treatment of the different cell lines with VitE-ISDs did not cause significant changes in the levels of Bcl-2 and Bax in PC3 and LNCaP cells. Furthermore, no change in the levels of AIF protein were detected following the treatment of cells with the different test compounds (Fig. 9).

Induction of Caspase-Dependent and -Independent DNA Damage by VitE-ISDs

In order to further investigate the mechanism of induction of cell death by VitE-ISDs, we performed comet assays. The results of these experiments have shown that all compounds were capable of inducing DNA damage in the 3 cell lines examined (Fig. 10). In general, δ-TT and TPGS-e were the most effective isofrom and synthetic derivative, respectively, with regard to their ability to induce DNA damage in the DU145 (Fig. 10A), PC3 (Fig. 10B), and LNCaP (Fig. 10) cell lines. Overall the
oxidative damage caused was as follows: TPGS-e > TPGS > \(\delta\)-TS > \(\delta\)-TT > \(\gamma\)-TT (Fig. 10).

In order to further investigate the dependency of DNA damage on caspase activation, comet assays were performed following the incubation of the compounds in the 3 different cell lines in the presence or absence of the caspase inhibitor z.vad.fmk. The results of these experiments showed that in the AR+ LNCaP cells, the isoforms \(\gamma\)-TT and \(\delta\)-TT induced caspase-dependent DNA damage, while the synthetic derivatives \(\gamma\)-TS, \(\delta\)-TS, TPGS, and TPGS-e induced caspase-independent DNA damage (Fig. 11C). On the other hand, in AR- cell lines the DNA damage induced by all compounds was caspase-independent (Fig. 11A and 11B).

**DISCUSSION**

In this study, we examined the molecular events activated following treatment of AR- and AR+ prostate cancer cell lines with 2 isoforms (\(\gamma\)-TT, \(\delta\)-TT) and 4 synthetic derivatives of
FIG. 5. A: DU145, PC3, and LNCaP cells were incubated with 20 µM γ-tocotrienol (γ-TT), δ-tocotrienol (δ-TT), γ-tocotrienyl succinate (γ-TS), δ-tocotrienyl succinate (δ-TS) or 100 µg/ml Etoposide for 72 h. The cells were extracted and equal amounts of protein were analyzed by Western blotting for the detection of full-length (116 kDa) and cleaved (89 kDa) poly(ADP ribose) polymerase (PARP) following the incubations. The membranes were stripped and reprobed for α-tubulin and the latter was used as loading control. B: Densitometry was performed for (A) to detect the levels of full-length and cleaved PARP in (i) DU145, (ii) PC3, and (iii) LNCaP cells. The results are representative of 3 different experiments.

vitamin E (γ-TS, δ-TS, TPGS, TPGS-e). The synthetic derivatives were investigated in order to determine whether any of these compounds were more effective than the natural isoforms of vitamin E with regard to their ability to induce cell death.

The results of the current investigation suggest that of all VitE-ISDs examined δ-TT and TPGS-e were the most effective isoform and synthetic derivative respectively with regard to their ability to induce cell death in 3 prostate cancer cell lines. In particular, TPGS-e was included in this investigation because
FIG. 6. A: DU145, PC3, and LNCaP cells were incubated for 48 h with 10 µM or 20 µM of α-tocopheryl polyethylene glycol succinate (TPGS), 10µM α-tocopheryl polyethylene glycol ether (TPGS-e), and 20 µM of TPGS-e and in the presence or absence of 20 µM z.vad.fmk. The LNCaP cells were also incubated with 100 µg/ml Etoposide for 6 h. At the end of the incubation periods, the cells were extracted and equal amounts of protein (70 µg) were analyzed by Western blotting for the detection of (A) (i) poly(ADP ribose) polymerase (PARP). Densitometry was performed for (A) (i) to detect the levels of full-length and cleaved PARP in (A) (ii) DU145, (A) (iii) PC3 and (A) (iv) LNCaP cells. The results are representative of 3 different experiments. Western blotting was also performed for the detection of (B) caspases-3, -8, and -9. The membranes were stripped and reprobed for α-tubulin, and the latter was used as loading control.
FIG. 7. DU145, PC3, and LNCaP cells were incubated with 20 µM γ-tocotrienol (γ-TT), δ-tocotrienol (δ-TT), γ-tocotrienyl succinate (γ-TS), δ-tocotrienyl succinate (δ-TS), α-tocopheryl polyethylene glycol succinate (TPGS), and α-tocopheryl polyethylene glycol ether (TPGS-e) for 72 h. The cells were extracted and equal amounts of protein were analyzed by Western blotting for the detection of Bcl-2. The membranes were stripped and reprobed for α-tubulin and the latter was used as loading control.

in this novel compound the polyethylene glycol succinate is linked to α-TOC via an ether linkage instead of an ester linkage as in the case of TPGS (Fig. 1). Therefore, contrary to the ester linkage existing in the popular compound α-TOS (30,33) as well as in the rest of the synthetic derivatives γ-TS, δ-TS, and TPGS used in the current study (Fig. 1), the ether linkage of TPGS-e is not broken down by esterases of the intestinal tract, providing a possible advantage for use of this compound in the clinic. Interestingly, this study has provided some preliminary evidence that TPGS-e may be a more effective antitumor agent than the other VitE-ISDs examined. In particular, TPGS-e induces higher levels of DNA damage, caspase-3 activity, and CI-PCD than the rest of the compounds examined. Therefore, the potency of TPGS-e as a candidate chemotherapeutic agent should be the focus of further investigation.

A very interesting finding of the current study is the observation that VitE-ISDs induce CI-PCD. The induction of CI-PCD by VitE-ISDs is supported by evidence in the literature. For example, it has been shown that the induction of cell death in LNCaP prostate cancer cells by γ-TOC can only be mildly
Induction of Caspase-Independent Cell Death by Vitamin E

FIG. 9. A: DU145, PC3, and LNCaP cells were incubated with 20 µM γ-tocotrienol (γ-TT), δ-tocotrienol (δ-TT), γ-tocotrienyl succinate (γ-TS), or δ-tocotrienyl succinate (δ-TS) for 72 h. B: DU145, PC3, and LNCaP cells were incubated with 10 µM or 20 µM α-tocopheryl polyethylene glycol succinate (TPGS) or 20 µM α-tocopheryl polyethylene glycol ether (TPGS-e) in the presence or absence of 20 µM z.vad.fmk for 48 h. The cells were extracted and equal amounts of protein were analyzed by Western blotting for the detection of AIF. The membranes were stripped and reprobed for α-tubulin, and the latter was used as loading control. The results are representative of 3 different experiments.

Protected by incubation with the caspase inhibitor z.vad.fmk (25). Furthermore, in another study it was shown that caspase activation is not involved in the induction of apoptosis in MDA-MB-231 cells by γ-TT (26). Despite the induction of morphological changes associated with apoptosis (11, and data not shown) in this study, it has been shown that cleavage of PARP is not induced by all of the VitE-ISDs examined (26). These results are consistent with the observations of a recent study by Wu et al. (41), in which induction of apoptosis in the renal cell carcinoma cell line ACHN by α-TOS was visible in the absence of caspase activation and could not be prevented via caspase inhibition, thereby indicating the induction of CI-PCD.

In the current study, the induction of CI-PCD by VitE-ISDs is supported by the observation that VitE-ISDs induce cell death without activation of the effector caspase-3 in AR- cell lines DU145 and PC3 (Figs. 2 and 3). Further evidence for the activation of CI-PCD by VitE-ISDs is supported by the observation that, despite the activation of caspase-3 activity by several VitE-ISDs in AR+ LNCaP cells (Fig. 3), the induced cell death is not rescued by the caspase inhibitor z.vad.fmk (Fig. 2). Finally, the decrease in the levels of the full-length 116 kDa PARP in the absence of appearance of 89 kDa cleavage product (Figs. 5 and 6) suggests the cleavage of the protein by a caspase-independent enzyme. Therefore, the caspase-independent cleavage of PARP is consistent with the observed caspase-independent induction of cell death. The caspase-independent cleavage of PARP has been previously reported (42). Even though the enzyme responsible for the cleavage of PARP has not been identified, it is possible that the calpains may be directly or indirectly involved since these enzymes were reported to produce the caspase-independent cleavage of PARP (43). The enzyme involved in the cleavage of PARP is likely to have an important role in the induction of CI-PCD, and therefore its identification will contribute significantly toward our understanding of the signaling pathways and mechanisms regulated by VitE-ISDs. It is also possible that the caspase-independent events induced by VitE-ISDs are regulated by the release of cathepsins by lysosomes (44). Evidence to support this has been provided by Neuzil et al. (45), who showed that cells deficient in cathepsin D are resistant to treatment with α-TOS. Therefore, VitE-ISDs may induce lysosomal destabilization thereby causing the release of cathepsins to the cytoplasm. The role of cathepsins in the induction of CI-PCD have been previously reported (24,46).

The induction of CI-PCD by VitE-ISDs reported in this study is associated with the induction of both caspase-dependent and -independent DNA damage (Figs. 9–10). The induction of DNA damage by γ-TT in SGC-7901 adenocarcinoma cells has also been reported (45), but apoptosis in this cell line has been shown to be regulated by caspase-3 and to involve the downregulation of Bcl-2 (47). Interestingly, in the current study, the prevention...
FIG. 10. A: DU145, (B) PC3, and (C) LNCaP cells were incubated with 20 μM γ-tocotrienol (γ-TT), δ-tocotrienol (δ-TT), γ-tocotrieny1 succinate (γ-TS), or δ-tocotrieny1 succinate (δ-TS), 10 μM or 20 μM α-tocopheryl polyethylene glycol succinate (TPGS) or α-tocopheryl polyethylene glycol ether (TPGS-e) for 24 h. Comet assays were performed as described in the Materials and Methods. The levels of DNA damage induced by each compound in each different cell line are shown in a graphic representation. The treatments were performed in duplicate and represent the mean ± SEM of 3 different experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.001. (Continued)
of DNA damage induced by γ-TT and δ-TT in LNCaP cells by the caspase inhibitor z.vad.fmk does not prevent cell death, suggesting that caspase-mediated DNA damage is not a requirement for CI-PCD (Fig. 2A and 2B, Fig. 8C). Nevertheless, since the enzyme causing the caspase-independent DNA damage has not yet been identified, an inhibitor of this enzyme cannot be used to determine with certainty whether this event is a requirement for CI-PCD.

Apoptosis is regulated by the balance between antiapoptotic and proapoptotic proteins (48). The levels of Bcl-2, Bax, and AIF, which have previously been shown to be involved in the induction CI-PCD (24) were evaluated in the present study. High levels of the antiapoptotic protein Bcl-2 inhibit the induction of the classical apoptotic pathway (CD-PCD), whereas an increase in the levels or a translocation of proapoptotic proteins such as Bax from the cytoplasm to the mitochondrion may induce the release of cytochrome c and therefore cause induction of CD-PCD (24). AIF is commonly regulated during CI-PCD (24). The release of AIF from the mitochondrion requires an increase in the permeability of the external mitochondrial membrane and the cleavage of the full-length 67 kDa protein to a 57 kDa product that is released from the mitochondrion and translocates to the nucleus (49,50). The results of our study have shown that VitE-ISDs do not induce changes in the levels of Bcl-2, Bax, or AIF (Figs. 7–9). Similar observations have been reported by Takahashi and Loo (26), who showed that even though the incubation of MDA-MB-231 cells with γ-TT induces the appearance of morphological features of apoptosis, the isoform does not cause changes in the levels of Bcl-2 or Bax. Nevertheless, we have found substantial differences in the endogenous levels of Bcl-2 and Bax among the different cell lines we examined (Figs. 7 and 8). These observations suggest that the cellular microenvironment may therefore be critical for the induction of a particular type of cell death. Differences in the levels of Bcl-2 among the 3 different cell lines have been previously reported by others (51,52).

Recent evidence suggests that the apoptotic effects of VitE-ISDs may also be mediated through endoplasmic reticulum stress. γ-TT has been shown to induce +SA mammary tumor cell death, and this effect was associated with a corresponding increase in PARP-cleavage and activation of protein kinase-like endoplasmic reticulum kinase/eukaryotic translational initiation factor/activating transcription factor 4 (PERK/eIF2α/ATF-4) pathway, a marker of ER stress response. This treatment also caused a large increase in C/EBP homologous protein (CHOP) levels, a key component of ER stress-mediated apoptosis that increases expression of tribbles 3 (TRB3) (53). γ-TT has also been found to induce ER-stress activation of c-Jun NH2-terminal kinase (JNK) and p38 MAPK, followed by upregulation of DR5 in a CHOP-dependent manner in human breast cancer cells. Additional analyses showed that γ-TT activated JNK and p38 MAPK and upregulated death receptor 5 (DR5)
FIG. 11. (A) DU145, (B) PC3, and (C) LNCaP cells were incubated with 20 $\mu$M z.vad.fmK, $\gamma$-tocotrienol ($\gamma$-TT), $\delta$-tocotrienol ($\delta$-TT), $\gamma$-tocotrienyl succinate ($\gamma$-TS), or $\delta$-tocotrienyl succinate ($\delta$-TS), $\alpha$-tocopheryl polyethylene glycol succinate (TPGS), or 10 $\mu$M $\alpha$-tocopheryl polyethylene glycol ether (TPGS-e) in the presence or absence of 20 $\mu$M z.vad.fmK for 24 h. Comet assays were performed as described in the Materials and Methods. The levels of DNA damage induced by each compound in each different cell line are shown in a graphic representation. The treatments were performed in duplicate and represent the mean ± SEM of 3 different experiments. $^*P$ value < 0.05, $^{**}P$ value < 0.01, $^{***}P$ value < 0.001, $^{****}P$ value < 0.0001.

and CHOP. Both DR5 and CHOP upregulation were required for $\gamma$-TT-induced apoptosis, and DR5 was transcriptionally regulated by CHOP after $\gamma$-TT treatment (54).

$\alpha$-TEA (RRR-$\alpha$-tocopherol ether-linked acetic acid analog), a derivative of RRR-$\alpha$-tocopherol (vitamin E), exhibits anti-cancer actions in vitro and in vivo in a variety of cancer types. $\alpha$-TEA induces ER stress-dependent increases in death mediators JNK/CHOP/DR5 and decreases in survival mediators c-FLIP-L and Bcl-2 in human breast cancer cells. These ER stress mediated events function downstream of $\alpha$-TEA triggered TRAIL/DR5/caspase-8 signaling, leading to upregulation of JNK, CHOP, and DR5 and downregulation of c-FLIP and Bcl-2 (55). These studies suggest that VitE-ISDs should be investigated further for their ability to induce apoptosis through ER-mediated stress.

In general, the results of the current study have shown that VitE-ISDs induce caspase-dependent and -independent DNA damage and dominant CI-PCD. The decision on whether caspase activity and caspase-dependent DNA damage are induced or not is dependent on the structure of the particular VitE-ISD [e.g., $\gamma$-TT and $\delta$-TT induce caspase-dependent DNA damage, whereas TPGS and TPGS-e induce caspase-independent DNA damage in LNCaP cells (Figs. 9–10)] as well as on the cellular microenvironment [e.g., $\gamma$-TT, $\delta$-TT, TPGS, and TPGS-e induce caspase-3 activity in AR+ LNCaP cells but not in AR- DU145 and PC3 cells (Fig. 2)]. These results therefore signify that the apoptotic pathway that prevails with each VitE-ISD depends on the tissue context, and the AR may play a crucial role in this. The importance of the cellular microenvironment on the induction of caspase-dependent and -independent signaling pathways is also obvious by the observation that the DNA damaging agent etoposide induces caspase-3 activity in LNCaP and DU145 but not in PC3 cells (Fig. 4).

Overall, the results of this investigation suggest that the capacities of $\delta$-TT and TPGS-e to promote CI-PCD in prostate cancer cell lines may find applications in prostate cancer treatment. For example, it is possible that the combination of the vitamin E isoform $\delta$-TT or the synthetic derivative TPGS-e with conventional chemotherapeutic agents may lower the levels of tumor resistance and provide new treatment options to prostate cancer patients. The results encourage further testing of the most promising of these compounds in preclinical models of prostate cancer.

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