



Original Contribution

Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress

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ABSTRACT

Although the metabolism of vitamin E has been extensively studied in cell culture, animals, and humans, biochemical analyses of intermediate metabolites are scarce. We here describe the synthesis and proapoptotic properties of long-chain metabolites of α - and δ -tocopherol. Several long-chain vitamin E metabolites, namely 13'-hydroxy- and 13'-carboxychromanols, were synthesized from garcinoic acid, a δ -tocotrienol derivative extracted from the African bitter nut *Garcinia kola*. Both α - and δ -13'-carboxychromanol induced cell death in HepG2 cells at EC₅₀ of 13.5 and 6.5 μ M, respectively. Apoptosis was quantified by annexin V/7-AAD staining and flow cytometry analysis. By immunoblot analyses, we observed activation of both caspase-3 and caspase-9 as well as PARP-1 cleavage. Parameters of mitochondrial dysfunction including reduced mitochondrial membrane potential and increased intracellular and intramitochondrial reactive oxygen species formation were observed after metabolite treatment. Last, long-chain hydroxychromanols were readily metabolized to the corresponding carboxychromanols in HepG2 cells. Taken together, these results indicate that long-chain metabolites may be responsible for antiproliferative properties of vitamin E vitamers.

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The term "vitamin E" describes a group of lipophilic chain-breaking antioxidants of plant origin. The vitamin E molecule consists of a chromanol head group and an isoprenoid side chain. Dependent on the methylation pattern of the chromanol ring and the number of double bonds of the isoprenoid side chain, the vitamers are classified as α -, β -, γ -, or δ -tocopherol (α -, β -, γ -, or δ -T) or tocotrienols (α -, β -, γ -, or δ -T3). Since the discovery of vitamin E, research focused primarily on the antioxidant properties of α -tocopherol, whereas new findings have shown additional functions of other vitamers and their corresponding metabolites [1].

In 1995, Schulz et al. [2] identified α -carboxyethylhydroxychromanol (α -CEHC) as a water-soluble glucuronide conjugate of α -T in the urine and designated α -CEHC as a marker for adequate vitamin E intake. All vitamin E isomers are metabolized via oxidative degradation of their side chain. This process is initiated by ω -hydroxylation through CYP3A4 and/or CYP4F2, leading to 13'-hydroxychromanols (13'-OH), and α -oxidation of the 13'-carbon atom, which results in 13'-carboxychromanols (13'-COOH) (Supplemental Fig. 1) [3,4]. The breakdown of the side chain through subsequent β -oxidation steps

generates intermediate long- and short-chain carboxychromanols of which CEHC and conjugates thereof are the final metabolites. The rate of degradation depends on the methylation at the C5-atom of the chromanol ring and the saturation of the side chain. Therefore the conversion of γ -T or α -T3 is much more effective than that of α -T [4,5].

Specific functions of the short-chain metabolites were discovered in the 1990 s. These differed remarkably from the activities of the corresponding tocopherols. Wechter et al. [6] identified γ -CEHC as an endogenous natriuretic factor. This activity is mediated by the inhibition of the 70 pS K⁺ channel in the apical membrane of the thick ascending limb of the kidney [6]. Furthermore, Conte et al. [7] demonstrated antiproliferative effects of γ -CEHC in LNCaP cells. In 2004, Hensley et al. [8] showed anti-inflammatory effects of α - and γ -CEHC in TNF- α - or bacterial lipopolysaccharide-treated rat aortic endothelial cells and mouse microglial cultures through inhibition of PGE₂ and nitrite production.

Functions of long-chain vitamin E metabolites are widely unexplored. Jiang et al. [9] found that 13-carbon-long carboxychromanols are potent inhibitors of cyclooxygenase-1 via binding interactions with the substrate-binding site of COX-1. Zhao et al. [10] found long-chain metabolites (13'-COOH) in human fecal samples 48 h after supplementation with a single dose (1.6 g) of α -T.

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Recently, α - and δ -13'-OH and α - and δ -13'-COOH metabolites were semisynthesized from garcinoic acid, a constituent of the African bitter nut *Garcinia kola*, and thus for the first time became available for biochemical characterization [11].

In this study, we examined the induction of apoptosis by the long-chain tocopheryl metabolites α -tocopherylic acid, δ -tocopherylic acid, and their corresponding alcohols in human hepatoma (HepG2) cells.

Experimental procedures

Chemical synthesis

All chemicals were used as received from the supplier. Antimycin A, PtO₂, LiAlH₄, and SnCl₂ were obtained from Sigma-Aldrich (Schneidorf, Germany). Diethyl ether, methanol, and chloroform were obtained from Roth (Karlsruhe, Germany). α -CEHC, δ -CEHC, and 5'-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (α -CMBHC) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). *G. kola* seeds were obtained from AnalytiCon Discovery (Potsdam, Germany). Thin-layer chromatography analysis was performed on Silica Gel 60 F254-coated plates from Merck (Darmstadt, Germany). ¹H NMR and ¹³C NMR spectra were recorded at 300 and 75.47 MHz, respectively, on a Bruker AMX 300 (Bremen, Germany) with CDCl₃ as solvent and trimethylsilane as internal standard. Electrospray ionization mass spectrometry (ESI-MS) was performed in negative ionization mode on a MAT 95 XL-Trap (Thermoquest Finnigan, Bremen, Germany).

Isolation of garcinoic acid and metabolite synthesis

Garcinoic acid (δ -tocotrienoloic acid) was isolated from the African bitter nut *G. kola* according to published procedures with slight modifications [12]. In brief, 1 kg of seeds was mashed and extracted three times with 1 L of methanol. The solvent was evaporated and the residue was dissolved in a mixture of methanol and chloroform (95/5 vol/vol), dried with sodium sulfate, and evaporated to produce 13.3 g of a crude extract. The extract was dissolved in 30 ml of methanol/chloroform (95/5 vol/vol) and applied to a silica-gel column (150 g) to isolate a crude product with an *R_f* of 0.18. Rechromatography of the crude product on silica gel using a mixture of hexane and acetone (65/35 vol/vol) gave 3.8 g of garcinoic acid (purity >95%). The compound was characterized by NMR and mass spectroscopy (see supplemental data). 13'-Metabolites of α - and δ -tocopherol were synthesized according to the method described by Mazzini et al. [11]. In brief, hydrogenation of the double bonds of garcinoic acid under PtO₂ catalysis, at room temperature, yielded 13'-(6-hydroxy-2,8,-dimethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid (δ -13'-COOH or δ -tocopherylic acid). SnCl₂-catalyzed methylation of the chromanol ring afforded 13'-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid (α -13'-COOH or α -tocopherylic acid). Corresponding 13'-hydroxychromanols were obtained via reduction with LiAlH₄. All metabolites were characterized by NMR and mass spectroscopy and were in accordance with published data (see supplemental data). Purity of synthesized metabolites was >95% (HPLC).

Cell culture conditions

HepG2 cells were maintained in RPMI 1640 medium (Pan-Biotech, Aidenbach, Germany) containing 10% (vol/vol) FBS and 1% (vol/vol) antibiotic/antimycotic solution (100 U/ml penicillin and 10 μ g/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37 °C. Hepatocytes were grown to 80% confluence on 15-cm cell culture dishes. For experimental procedures, cells were rinsed with PBS, trypsinized, and transferred to a Falcon tube. After centrifugation, cells were quantified, diluted with medium, and seeded either into 96-well

microtiter plates (20,000–40,000/well) or into cell culture dishes (8 \times 10⁶/dish) for metabolism studies. After 24 h stock solutions of substances were diluted in prewarmed RPMI medium to desired concentrations. To obtain a uniform solvent concentration, an equal volume of dimethyl sulfoxide (DMSO) was also added to the medium of control samples. Cells were then incubated with prepared solutions for certain periods of time at the conditions indicated.

Examination of EC₅₀ by sulforhodamine B assay

To determine cytotoxic effects, 30,000 cells per well were seeded in a 96-well plate (Greiner, Germany) in RPMI medium as described above. After 24 h the medium was changed to RPMI containing 0.5% FBS. Cells were then exposed to various concentrations (0.005–50 μ M) of α - or δ -tocopherol or their corresponding acids or alcohols for 24 h. Cells were fixed with 10% trichloroacetate for 1 h at 4 °C. Sixty microliters of a 0.4% solution of SRB (sulforhodamine B; Sigma) in 1% acetic acid was given to each well. After several washing steps with 1% acetic acid, the protein-bound SRB was dissolved in 10 mM Tris base solution for photometric determination at 560 nm using an Optima microplate reader (BMG Labtech, Offenburg, Germany).

Analysis of apoptosis by flow cytometry

The flow cytometry system Cell Lab Quanta SC-MLP 1.0 (Beckman Coulter, Germany) and Annexin V-FITC/7-AAD-kit (Beckman Coulter) were used according to the manufacturer's instructions. Therefore, 4.5 \times 10⁶ cells were cultured in the presence of control medium or 10 μ M α - or δ -tocopherol or their corresponding acids or alcohols for 6 h. After being washed with PBS, the cells were detached with trypsin and 5 \times 10⁵ cells of each group were resuspended in binding buffer and incubated with annexin V-FITC/7-AAD for 15 min on ice. Dead cells and debris were excluded by selective gating based on electronic cell volume. Thirty thousand events were collected from each sample. By using dual-parameter staining, early and late apoptosis was quantified, using the Quanta Analysis software (Beckman Coulter). In preliminary experiments, unstained medium-treated cells were used to define living cells (lower left quadrant). Cells treated with DMSO as control were singly stained with annexin V-FITC or 7-AAD to define respectively the lower right and upper quadrants.

Western blot analysis

Cells were lysed in Soerensen buffer containing EDTA and a phosphatase/protease-inhibitor cocktail (Na-vanadate, NaF, and phenylmethylsulfonyl fluoride; Sigma). The solution obtained was sonicated and denatured by boiling in Laemmli buffer for 5 min at 95 °C. Protein samples (25 μ g per lane) were then separated by 10 or 16% SDS-PAGE [13]. After electrophoresis, proteins were transferred to nitrocellulose membrane (Millipore). After being stained with Ponceau red, the membranes were blocked (PBS containing 0.1% Tween and 5% skimmed milk) and incubated overnight with rabbit polyclonal anti-caspase-3, rabbit polyclonal anti-cleaved caspase-3, rabbit polyclonal anti-poly-ADP ribose polymerase-1 (PARP-1), rabbit polyclonal anti-cleaved PARP-1, rabbit polyclonal anti-caspase-9, rabbit polyclonal anti-cleaved caspase-9, rabbit polyclonal anti-caspase-7, or rabbit polyclonal anti-cleaved caspase-7. All primary antibodies were diluted 1:1000 in 5% BSA/TBS/Tween (w/v). Blots were washed with 5% BSA/TBS/Tween and subsequently incubated with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:1000). Membranes were developed with a chemiluminescence kit (Amersham, Buckinghamshire, UK) following the manufacturer's instructions. Mouse monoclonal anti-tubulin antibody (1:1000) was used as a loading control. All antibodies used were purchased from Cell Signalling (Hertfordshire, UK).

Determination of reactive oxygen species (ROS) formation

To assess the total cellular amount of ROS, the DCF assay was conducted essentially as previously described [14,15]. In brief, HepG2 cells were loaded with 2',7'-dihydrodichlorofluorescein diacetate (H₂-DCF-DA; 100 μM final concentration) in RPMI medium for 30 min at 37 °C in an atmosphere of 5% CO₂. H₂-DCF-DA is a nonfluorescent fluorescein derivative that emits fluorescence after oxidation to 2',7'-dichlorofluorescein (DCF). Fluorescence intensity is directly proportional to intracellular ROS formation. After being loaded with H₂-DCF-DA, cells were washed with PBS, and test compounds were added for 6 h. Fluorescence intensity was measured using a NOVOstar microplate fluorimeter (BMG Labtech) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

A similar setup was used for the determination of mitochondrially derived ROS. Cells were loaded with MitoTracker CM-H2XROS (Molecular Probes/Invitrogen, Germany) and treated as described above. Fluorescence intensity was measured at an excitation wavelength of 543 nm and an emission wavelength of 590 nm [15].

Mitochondrial membrane potential

HepG2 cells were seeded in a 96-well microtiter plate and kept overnight. The staining solution was prepared by diluting a 10 mM JC-1 stock solution (Molecular Probes) 1:1000 with PBS. Cells were incubated with antimycin A (30 μM as positive control) or vitamin E metabolites (20 μM) in an atmosphere of 5% CO₂ and 37 °C for 1 h. Thereafter, the medium was removed; the cells were washed and incubated with the JC-1 staining solution for 6 h. Fluorescence was measured using an Optima microplate fluorimeter (BMG Labtech) at 535 nm for JC-1 monomers and at 590 nm for JC-1 aggregates [15].

Extraction and HPLC–UV analysis of metabolites

Supernatants of culture medium were extracted as described by Li et al. [17] with slight modifications and analyzed by reversed-phase (RP) HPLC [16–18]. In brief, we used an RP Kromasil C18 column (5 μm 250 × 3.0 mm) at a flow rate of 0.8 ml/min with the following gradient: 100% A (35% acetonitrile, 65% 10 mmol/L ammonium acetate at pH 4.3) for 8 min and then linearly increasing from 8 to 30 min to 100% B (96% acetonitrile, 4% 10 mmol/L ammonium acetate at pH 4.3). Buffer B was maintained for 15 min and then increased from 45 to 50 min to 100% C (tetrahydrofuran/acetonitrile 1/6 vol/vol). Buffer C was maintained for 15 min to elute tocopherols and lipids. Metabolites were quantified with UV detection at 293 nm.

Statistical analyses

EC₅₀ values were calculated using Origin 7.5 G software. Means and standard deviations were calculated using Microsoft Excel. Statistical analysis was performed with SPSS 15.0. Differences between two groups were assessed using an unpaired Student *t* test and two-tailed distribution, with *p* below 0.05 considered statistically significant and *p* below 0.01 highly significant.

Results

Semisynthesis, isolation, and characterization of tocopheryl metabolites

Long-chain tocopheryl carboxychromanols have been known as metabolic intermediates for several years [3,5,9,19]; however, only recently a semisynthetic approach was published based on garcinoic acid, a constituent of the African bitter nut *G. kola* [11]. To obtain a significant amount of starting acid, we have developed an optimized isolation procedure yielding 3.8 g of garcinoic acid derived from 1 kg of *G. kola* seeds (0.38% wt/wt). On the above obtained material we

performed the synthesis of α- and δ-13'-carboxychromanols and α- and δ-13'-hydroxychromanols obtaining sufficient amounts of metabolites for chemical and biochemical investigations. The synthetic procedure includes platinum oxide-catalyzed hydrogenation of the side-chain double bonds and methylation of the chromanol ring system to obtain α-13'-COOH. Reduction with LiAlH₄ afforded 13'-hydroxychromanols (Fig. 1). All synthesized metabolites were purified by silica-gel flash-chromatography obtaining products with purities >95% (according to HPLC, data not shown). Metabolites were characterized by ¹H and ¹³C NMR and ESI-MS (see supplemental data).

Antiproliferative effects on HepG2 cells

Earlier studies suggested potential proapoptotic properties of medium- and long-chain carboxychromanol metabolites [7,11,20]. Consequently, we investigated the potential antiproliferative effects of the aforementioned metabolites via sulforhodamine B assay, commonly used for the screening of natural products [21]. HepG2 cells were used in the biochemical assays because their tocopherol metabolism parallels that found in humans [4]. Cells were starved for 16 h and incubated with metabolites for 24 h to determine EC₅₀ values. EC₅₀ curves of metabolites are depicted in Figs. 2A–F. The effective concentrations to arrest cell growth (50%) followed the order δ-13'-COOH (6.5 μM) ≈ α-13'-COOH (13.5 μM) << δ-13'-OH (>50 μM) < α-13'-OH, α- and δ-T (>100 μM). It seems noteworthy that carboxylation of the side chain is required to render tocopherols potent antiproliferative metabolites.

Induction of apoptotic cell death

We applied annexin V–FITC/7-AAD staining in combination with flow cytometry to differentiate between apoptosis and necrotic cell death. After incubation of HepG2 cells with 20 μM tocopherols or metabolites for 6 h, we co-incubated the cells with FITC-labeled annexin V as an early marker for phosphatidylserine externalization at the cell membrane. Early apoptotic events are located in the lower right quadrant of the FACS diagrams, whereas late apoptosis is found in the upper right quadrant (Fig. 3A). Addition of early and late apoptotic events revealed a significant increase in apoptosis compared to DMSO solvent control when cells were treated with long-chain carboxy-metabolites, particularly with α-13'-COOH (Fig. 3B).

Programmed cell death is associated with the induction of proteases, which finally leads to the disruption of cellular structures. We prescreened caspase-2, -3, -8, and -9 activities in a 96-well colorimetric caspase assay of 13'-carboxychroman-treated HepG2 cells. Both metabolites, α-13'-COOH and δ-13'-COOH, induced strong activation of caspase-3 and a slight increase in caspase-2 activity after 6 h at 20 μM (data not shown). Caspase-3 is one of the key executioners of apoptosis; and it is triggered via endoplasmic reticulum stress, extracellular stimuli, or mitochondrial ROS. Because caspase-8 activity remained unchanged in this assay, we postulated an intrinsic, mitochondria-initiated pathway of apoptosis. To further elucidate a potential mitochondrial involvement we determined full-length and cleaved caspase-3, -7, and -9 by Western blot analysis of cells treated with metabolites (20 μM) for 6 h. We found a pronounced activation of caspase-3, -7, and -9 by 13'-carboxychromanols and a less pronounced activation by δ-13'-OH. No activation was observed with tocopherols or α-13'-OH (Figs. 4A–C).

Because caspase activation induces cleavage of PARP-1, we additionally examined the cleavage of this protein by Western blot analysis. PARP-1 cleavage induced by metabolites parallels the activation of caspases as described above. In particular, 13'-carboxychromanols strongly induced cleavage of PARP-1 (Fig. 4D).

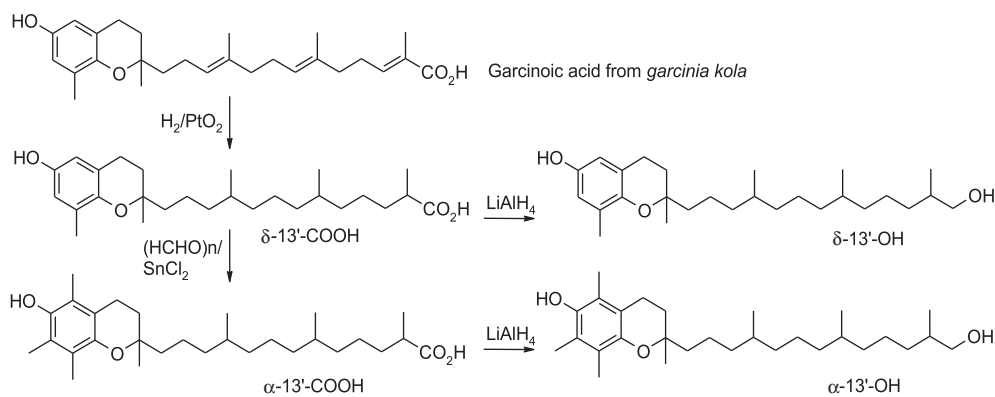


Fig. 1. Synthesis of the vitamin E metabolites investigated. Metabolites were semisynthesized starting from garcinoic acid, a tocotrienol derivative extracted from *G. kola*. Platinum oxide-catalyzed hydrogenation and subsequent methylation led to δ - and α -13'-COOH. Reduction of the acids afforded the alcohols δ - and α -13'-OH, respectively. All metabolites were purified >95% (according to HPLC). (Please see the supplemental data for chemical characterization of the compounds.)

Long-chain metabolites induce mitochondrial ROS and decrease mitochondrial membrane potential

Mitochondrial-derived apoptosis is associated with the generation of ROS. Consequently, we determined the generation of intracellular and intramitochondrial ROS by the tocol metabolites. We used the oxidative conversion of nonfluorescent $\text{H}_2\text{-DCF-DA}$ into fluorescent DCF for quantification of cellular stress as described by Wang and Joseph [22]. Incubation with $10\ \mu\text{M}$ 13'-carboxychromanols for 6 h induced a significant increase in ROS production in HepG2 cells (Fig. 5A). δ -13'-OH and α -13'-OH as well as both tocopherols did not affect ROS formation in the cells. After metabolite-treated cells were incubated with MitoTracker CM-H2XROS, a very similar picture was seen for intramitochondrial ROS formation. HepG2 cells were loaded with the nonfluorescent dye, which selectively accumulates within mitochondria and subsequently is oxidized to a fluorescent chromophore, as repeatedly shown in the past [15,23–25]. α - and δ -13'-COOH increased mitochondrial-derived ROS significantly (30–50% vs DMSO control, $p < 0.01$). Neither 13'-hydroxychromanol showed any significant ROS induction. Unexpectedly, a strong antioxidative effect

was seen for δ -T (35% reduction vs control, $p < 0.01$; Fig. 5B). This observation is in accordance with results from a recent in vitro study showing the highest antioxidative capacity for δ -T in the ORAC and CL assay [26]. However, in our setup we used nonphysiologically high amounts of δ -T ($20\ \mu\text{M}$), whereas human serum concentrations are below $1\ \mu\text{M}$. These results are in concordance with the Western blot analyses showing the strongest effects for the long-chain carboxychromanols in regard to induction of apoptosis.

We further investigated the mitochondrial membrane potential ($\Delta\Psi_m$), which is a reliable indicator of mitochondrial-dependent apoptosis and which is quantified by applying the fluorescent dye JC-1. In the presence of a high $\Delta\Psi_m$ the dye forms JC-1 aggregates with an emission wavelength of 590 nm [15,27]. We observed a minor reduction of $\Delta\Psi_m$ for α -13'-COOH and δ -13'-COOH at $10\ \mu\text{M}$ and a more significant effect at $20\ \mu\text{M}$ (60% reduction by α -13'-COOH vs control, $p < 0.01$, and 20% reduction by δ -13'-COOH vs control, $p < 0.01$; Fig. 5C). A significant loss of the membrane potential was also seen for δ -13'-OH (20% vs control, $p < 0.01$).

Taken together, these data explain the apoptosis-related results from the Western blot analyses and suggest that induction of ROS and

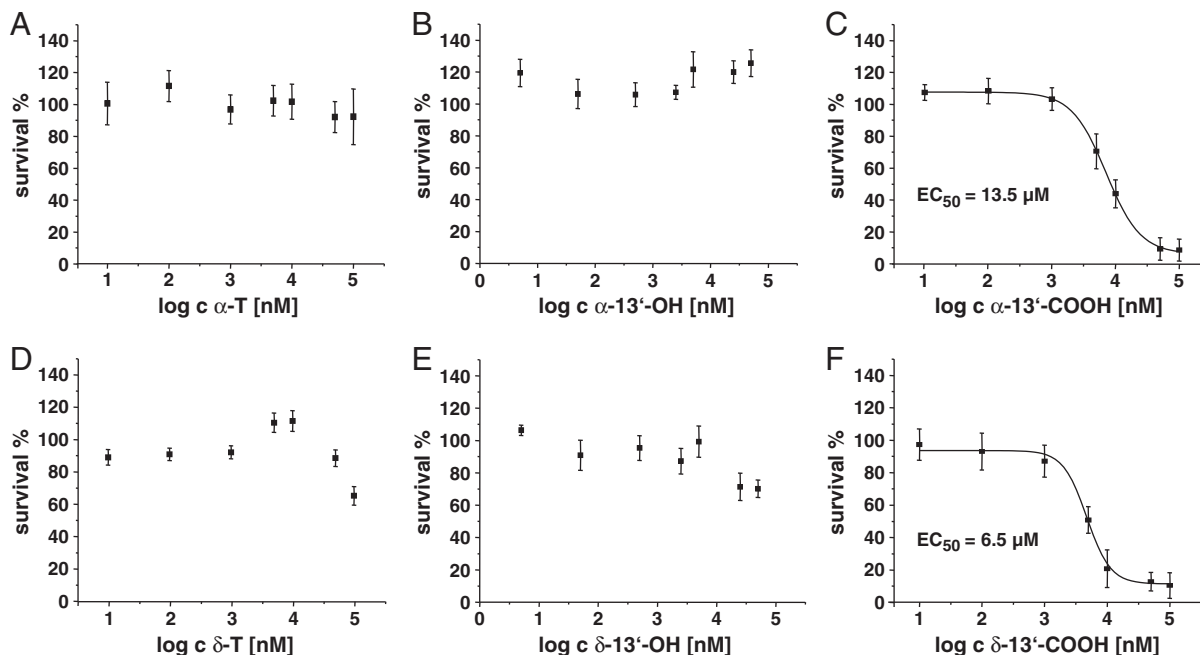


Fig. 2. Growth and survival of HepG2 cells after incubation with various tocopherol metabolites for 24 h. (A) α -Tocopherol, (B) α -13'-OH, (C) α -13'-COOH, (D) δ -tocopherol, (E) δ -13'-OH, and (F) δ -13'-COOH. Data are expressed as the means of three individual experiments \pm SD. EC_{50} values were calculated using Origin 7.5G software.

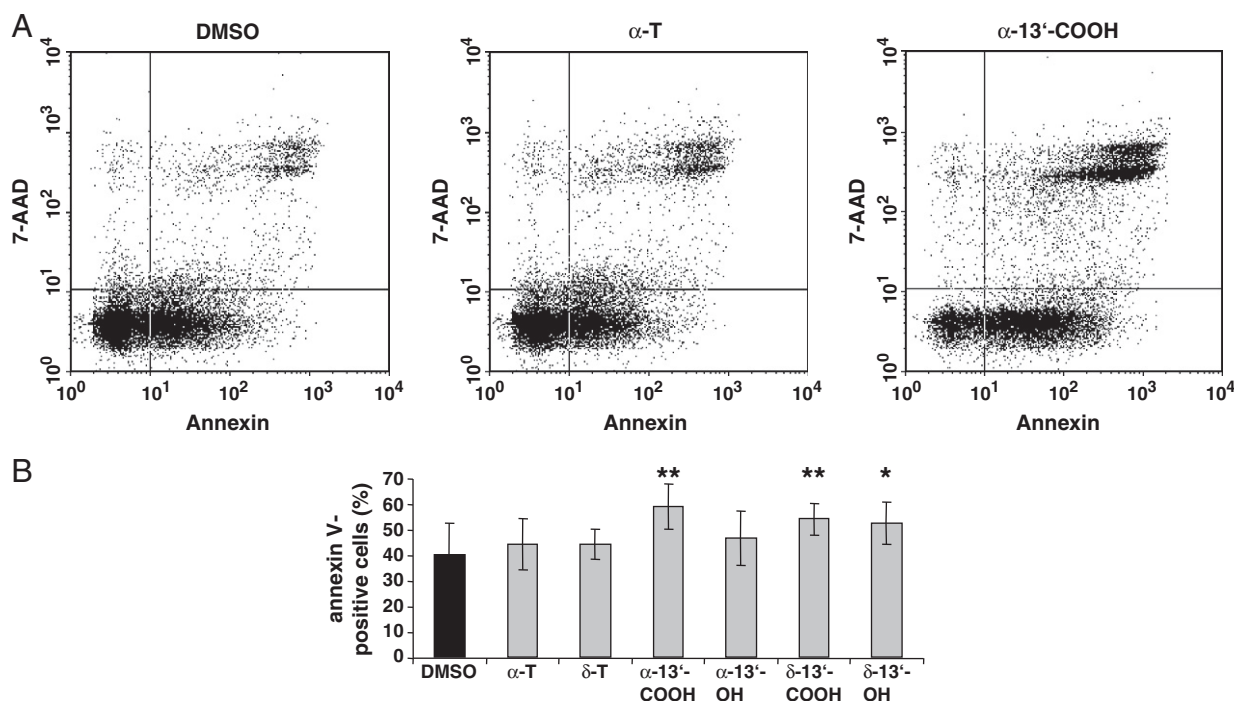


Fig. 3. Induction of apoptosis by vitamin E metabolites. Quantification of apoptosis by annexin V-FITC/7-AAD staining and flow cytometry (Cell Lab Quanta). (A) Representative FACS diagrams of HepG2 cells treated with the corresponding compounds for 6 h at 20 μ M. (B) Quantification of annexin V-positive cells. Data are expressed as means \pm SD of $n = 4$, * $p < 0.05$, ** $p < 0.01$ compared to DMSO.

reduction of $\Delta\Psi_m$ may explain the induction of apoptosis after exposure to long-chain vitamin E metabolites.

Metabolic conversion changes hydroxychromanols to proapoptotic carboxychromanols

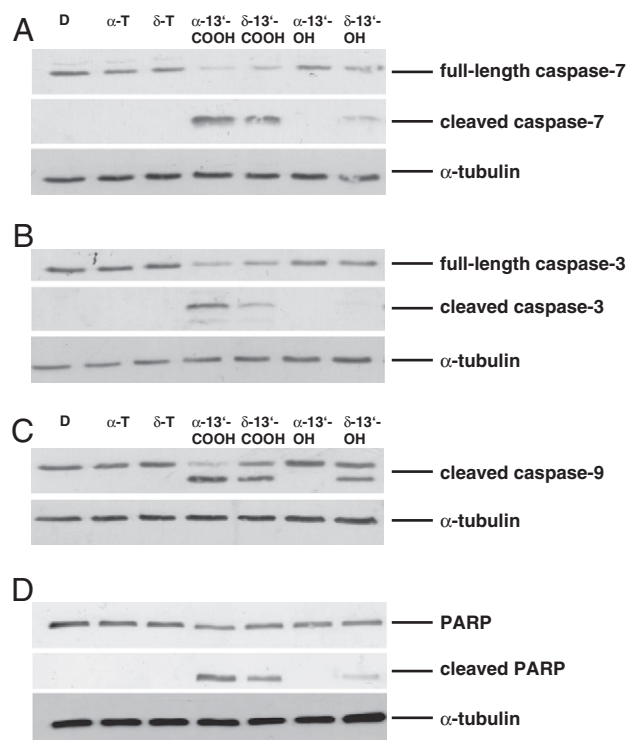


Fig. 4. Activation of caspases and PARP-1 by vitamin E metabolites. Representative expression levels of full-length and cleaved (A) caspase-7, (B) caspase-3, (C) caspase-9, and (D) PARP-1 in HepG2 cells using Western blotting. Cells were treated for 6 h with the corresponding compounds at 20 μ M. For each blot, α -tubulin expression was used as loading control.

Our data indicate that long-chain 13'-COOH are responsible for the induction of apoptosis in HepG2 cells. However, and in contrast to α -13'-OH, δ -13'-OH also induced apoptosis as shown by FACS analysis (Fig. 3) and Western blots (Fig. 4).

We therefore investigated the metabolic conversion of the alcohols (α -13'-OH and δ -13'-OH) into the more active carboxychromanols. We applied 50 μ M corresponding long-chain alcohol to HepG2 cells for 24 h. The cells were cultivated with 10% FBS to prevent apoptosis during the incubation time. The supernatant media of the cells were collected, extracted, and analyzed by HPLC-UV. Fig. 6A shows a representative HPLC chromatogram with the metabolites α -13'-OH, α -13'-COOH, and α -CMBHC (unknown metabolites are marked with an X). We quantified the known metabolites (Fig. 6B) according to standard curves (data not shown). Comparison of α -13'-OH and δ -13'-OH revealed a higher turnover rate to the corresponding acid for the δ -derivative. Thus, 75% of total metabolites (δ -13'-COOH and δ -CMBHC) evolved from δ -13'-OH after 24 h vs 60% of total metabolites (α -13'-COOH and α -CMBHC) produced from α -13'-OH. The corresponding 13'-COOH metabolites in the supernatant differed significantly ($22.7 \pm 1.4 \mu$ M α -13'-COOH vs $33.1 \pm 2.3 \mu$ M δ -13'-COOH; $p < 0.01$). Because HepG2 cells produce a higher amount of 13'-COOH (and presumably more medium- and short-chain metabolites) from δ -13'-OH, we postulate that they are more susceptible to the induction of apoptosis by δ -13'-OH than by α -13'-OH.

Discussion

A semisynthetic approach aimed at obtaining long-chain tocopherol metabolites enabled us to study their biological properties for the first time. We found that long-chain metabolites of α - and δ -T (α -13'-COOH and δ -13'-COOH) were strong inducers of apoptosis in HepG2 cells. FACS analyses and activation of caspase-9 and -3 suggest a mitochondrial-associated apoptosis pathway. Surprisingly, and in

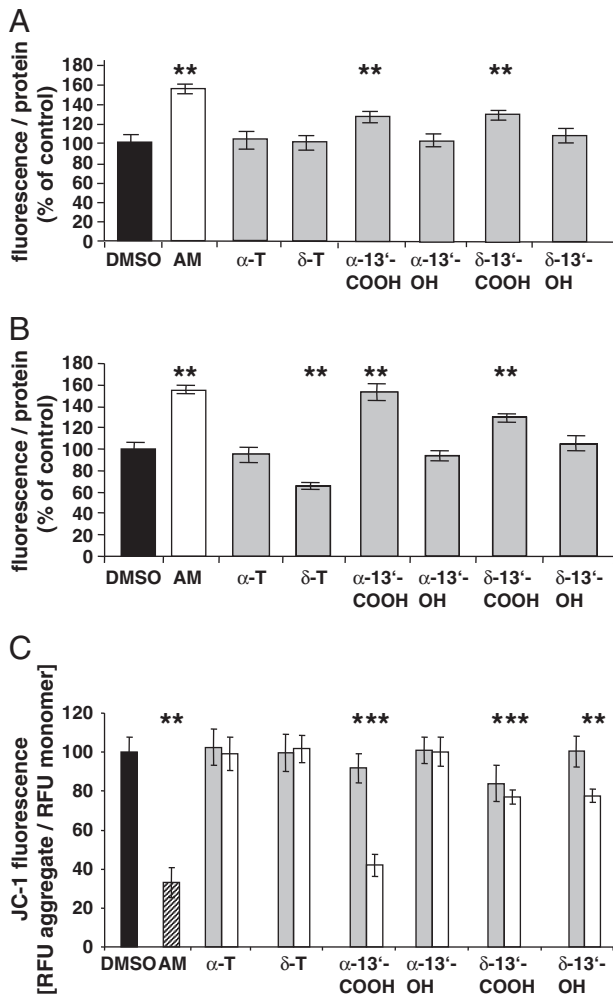


Fig. 5. Induction of ROS formation and reduction of mitochondrial membrane potential by vitamin E metabolites. (A) Direct comparison of cellular ROS formation in HepG2 cells after treatment with 10 μ M vitamin E and its metabolites. Cellular ROS were quantified by DCF. DMSO value was set to 100% and antimycin A (AM) was used as positive control. (B) Mitochondrial ROS formation in HepG2 cells after treatment with 10 μ M vitamin E metabolites and antimycin A (positive control). ROS were quantified by MitoTracker CM-H2XRos. (C) Influence of metabolites on mitochondrial membrane potential ($\Delta\Psi_m$) measured by JC-1 fluorescence. HepG2 cells were treated with 10 (gray bars) and 20 μ M (white bars) for 1 h and fluorescence of JC-1 monomers (em 535 nm) and aggregates (em 590 nm) was measured. The quotient of fluorescence values correlates with $\Delta\Psi_m$. Antimycin A (20 μ M) was used as positive control. All data are expressed as means \pm SD of $n=8$, * $p<0.05$, ** $p<0.01$, compared to DMSO.

contrast to α - and δ -T, long-chain carboxychromanols promoted intracellular and intramitochondrial ROS formation and reduced mitochondrial $\Delta\Psi_m$. Although the exact molecular mechanism remains to be established and warrants further investigation, only long-chain metabolites bearing a terminal carboxyl group were capable of inducing apoptosis. Moreover, we here could show that δ -13'-OH was partly converted to δ -13'-COOH in HepG2 cells and thereby induces apoptosis as well, although to a lesser extent.

According to these results we postulate an involvement of long-chain metabolites in the cancer-preventing properties of tocopherol and tocotrienols previously reported in the literature [28–32]. At least in HepG2 cells, the ability of tocotrienols and tocopherols to induce apoptosis parallels their catabolic rates. For example, earlier studies showed that HepG2 cells differentially metabolize tocopherols and tocotrienols [4,5]. Significant amounts of short-chain metabolites (CEHC and CMBHC) were found after incubation with γ - and δ -T, respectively, but extremely low concentrations after treatment with α - and β -T, respectively. High turnover rates were observed for

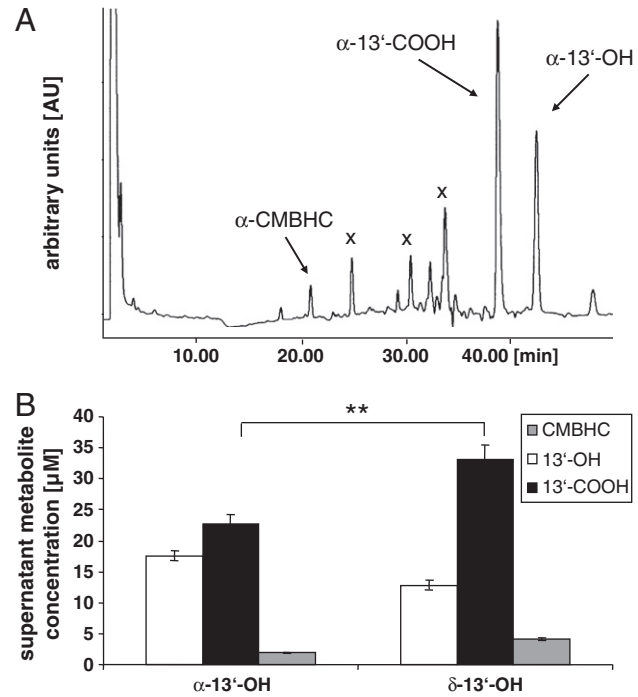


Fig. 6. Generation of tocopherol metabolites by human hepatoma cells. (A) Representative HPLC-UV chromatogram of α -T metabolites released from HepG2 cells. Cells were grown for 48 h in the presence of 10% FBS and 50 μ M/L α -13'-OH. Thereafter, the supernatant medium was collected and metabolites were extracted. X, unknown metabolite. (B) Quantification of supernatant metabolites after treatment with 50 μ M α -13'-OH or 50 μ M δ -13'-OH. All data are expressed as means \pm SD of $n=3$.

tocotrienols. Hence a combination of long-chain and short-chain intermediates of tocotrienols may induce apoptosis rather than tocotrienols themselves. This possibility requires additional attention in the case of tocopherols, because only γ - and δ -T induce apoptosis in several cell types, whereas α - and β -T have no effect at similar concentrations [33,34]. Cell proliferation was inhibited in CaCo-2 (colon carcinoma), DU-145, LNCap (prostate carcinoma), and SaOs-2 (osteosarcoma) cells when treated with 25 μ M γ -T, whereas α - and β -T were not effective [35]. Another study reported that γ -T significantly induced apoptosis in SW480, HCT-15, HCT-116, and HT-29 colon cancer cells, whereas α -T had no effect [34]. Because cytochromes (CYP3A4 and/or CYP4F2) are responsible for the initial activation of the side chain of tocols, we screened the literature for several of the cell types described above and found mRNA or protein data for CYP3A4 in CaCo-2 [36], HT-29 [37], SW480 [38], and SaOs-2 cells [39]. Data on CYP4F2 expression are scarce, especially for the cell types described. Unfortunately, tocopherol metabolism was not investigated in these studies and thus the differential effects of vitamins cannot be unambiguously attributed to specific metabolites.

Additional support for our hypothesis comes from a recent study by Jiang et al. [9]. The authors showed that γ -13'-COOH and γ -T could inhibit COX-2-catalyzed PGE₂ synthesis in IL-1 β -stimulated human lung adenocarcinoma cells (A549). The cellular inhibition of γ -T was partially diminished by sesamin, which blocks the metabolism of vitamin E. This suggests that mainly tocopherol metabolites may be responsible for the inhibitory effects, especially because A549 cells are known to have a high metabolic rate for tocopherols [19].

Recently, Mustacich et al. [40] found ω -hydroxylation products, namely α -13'-OH, in liver microsomes from rats receiving subcutaneous injections of 10 mg α -T/100 g body wt for 3 days. The authors postulated that β -oxidation steps of the α -T side chain do not exclusively occur in peroxisomes but also in mitochondria [40]. Accordingly, they found high amounts of α -CEHC in the mitochondrial compartment. Thus, 13'-carboxychromanols can enter the mitochondria presumably after

esterification with carnitine and via the CPT1 fatty acid transporter pathway. Possible targets within the mitochondria are the focus of recent cancer research, and chemopreventive anti-cancer agents that target the mitochondrial compartment are called "mitocans" [41,42]. A well-known candidate is a redox-silent vitamin E derivative, tocopheryl succinate, which is a strong inducer of apoptosis capable of entering the mitochondrial compartment to generate ROS by inhibition of complex II of the respiratory chain [43,44]. Moreover, Piericidin A is an inhibitor of complex I that produces ROS and, interestingly, shares structural similarities with 13'-COOH, such as an isoprenoid side chain with a polar modification [45]. Hence, and on a speculative basis, long-chain carboxychromanols may act by inhibiting the respiratory chain, thereby producing ROS. Further studies are warranted to answer this question.

Large intervention studies with vitamin E supplements faced several drawbacks and outcomes of these studies were overall disappointing [46–48]. Moreover, a recent meta-analysis revealed an increase in total mortality in the vitamin E intervention groups [49,50]. In this regard, it should be noted that α -T is known to be recycled in mammalian species at nutritional doses and thus escapes metabolic breakdown. However, fortified intake of tocopherols might produce critical amounts of long-chain metabolites and could prevent proliferation of cancer cells. It seems noteworthy that Zhao and colleagues found α -13'-COOH in feces of supplemented humans [10]. These authors postulated that after absorption of α -T, long-chain metabolites are generated in the liver and secreted via the bile to enter the enterohepatic circulation. According to this previous study and the results presented here, we suggest that vitamin E metabolism must be taken into consideration when future vitamin E intervention studies are planned, especially for the prevention of colorectal cancer.

Taken together, this study indicates that long-chain metabolites of vitamin E, and in particular tocopherylic acids, may be responsible for antiproliferative properties of vitamin E vitamers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2010.07.024.

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