Inhibition of UVB induced DNA photodamage in mouse epidermis by topically applied \(\alpha\)-tocopherol

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Ultraviolet B (UVB, 290–320 nm) exposure results in a variety of cellular insults including induction of cyclobutane pyrimidine dimers in DNA. Accumulation of these lesions can lead to mutations in critical genes and contribute to the development of nonmelanoma skin cancer. Topically applied \(\alpha\)-tocopherol (vitamin E) has previously been shown to prevent the induction of skin tumors in UVB irradiated female C3H/HeNTac mice. We hypothesized that \(\alpha\)-tocopherol, which absorbs strongly in the UVB, may act as a sunscreen to prevent photodamage. To explore possible mechanisms of photoprotection, we topically applied \(\alpha\)-tocopherol dispersed in a neutral cream vehicle to the dorsal epidermis of female C3H/HeNTac mice and exposed them to 2.5 J/m\(^2\)/s of UVB for 60 min. Immediately after exposure, we analyzed thymine dimer levels in DNA by capillary gas chromatography with electron capture detection. Epidermal DNA from mice receiving this UVB dose contained 247 \(\pm\) 42 pmol thymine dimers/µmol thymine. Topical application of \(\alpha\)-tocopherol inhibited dimer formation in a dose-dependent manner. A 1% \(\alpha\)-tocopherol dispersion inhibited the formation of thymine dimers to 43% of levels in vehicle controls. Several vitamin E compounds, including \(\alpha\)-tocopherol acetate, \(\alpha\)-tocopherol methyl ether, \(\gamma\)-tocopherol, and \(\delta\)-tocopherol also inhibited thymine dimer formation, but were five- to ten-fold less potent than \(\alpha\)-tocopherol. A variety of commercially available sunscreens were also less potent than \(\alpha\)-tocopherol in their ability to reduce dimer formation. These results suggest that DNA photoprotection is an important mechanism by which topically applied \(\alpha\)-tocopherol can inhibit UVB induced skin cancer. \(\alpha\)-Tocopherol acetate, the most common form of vitamin E in commercial skin care products, conferred less protection, perhaps due to its lower absorptivity in the UVB. Our results further underscore the importance of determining which forms of vitamin E can inhibit specific lesions involved in photocarcinogenesis.

Introduction

Ultraviolet B light (UVB\(^*\)) has been shown to be a complete skin carcinogen (1). Moreover, UVB induced DNA photodamage appears to be a critical event in photocarcinogenesis. Characteristic lesions in UVB irradiated DNA include cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts, which form between adjacent pyrimidines (2), and photohydrates. Pyrimidine dimers can produce deletion, frame shift, and substitution mutations and have been associated with distinctive C\(\rightarrow\)T and CC\(\rightarrow\)TT transitions in the genome (3). Many squamous and basal cell carcinomas contain such characteristic mutations within the p53 tumor suppressor gene (4–6).

In sunlight reaching the earth’s surface, it is the UVB portion which contains the most carcinogenic wavelengths. Thus, sunscreens act ideally scatter, reflect or absorb wavelengths in this range. Many commercial sunscreens contain compounds whose UV absorbances extend into the UVB, such as salicylates, cinnamates, benzophenones, and ester derivatives of 4-aminobenzoic acid (PABA). However, chemopreventive agents such as \(\alpha\)-tocopherol (\(\alpha\)TH, \(\lambda_{\text{max}}\) = 294 nm) also absorb in the UVB region. This absorbance may account in part for the ability of topically applied \(\alpha\)TH to suppress UVB induced damage such as cellular release of lysosomal enzymes (7), edema (8,9) and erythema (9). Topical d,l-\(\alpha\)-tocopherol acetate (\(\alpha\)TAc) has also been found to decrease lipid peroxidation and preserve normal replicative DNA synthesis in UVB irradiated hairless mice (10).

The efficacy of sunscreens is evaluated primarily on the basis of their ability to prevent erythema and edema (11). These manifestations of UVB exposure result from a number of secondary changes in the dermis and epidermis. Generation of reactive oxygen species (12), vascular changes (13), activation of inflammatory mediators and cells, membrane damage, and release of various cytokines (14) all contribute to these endpoints. From such a complex combination of events, it is difficult to attribute specific molecular protective effects to a sunscreen. Selected sunscreens have been shown to inhibit UV induced DNA damage (15–18), actinic damage (19), and photocarcinogenesis (20). Nevertheless, sunscreens are not routinely evaluated for their ability to prevent nonmelanoma skin cancers and the scope of their photoprotective actions at the molecular level remains largely undefined.

This investigation is based on the observation that topical application of \(\alpha\)TH prevented skin photocarcinogenesis, whereas application of the tocopherol derivatives \(\alpha\)TAc or \(\alpha\)-tocopherol succinate (\(\alpha\)TS) did not (21,22). This suggests a difference in the extent of protection provided against UVB damage by these closely related compounds and supports the idea that \(\alpha\)TH has substantial sunscreens properties. In order to investigate the hypothesis that \(\alpha\)TH can act as a sunscreen, we monitored the induction of a UV specific DNA photoproduct. Pyrimidine dimers are specific markers of UV-induced photodamage and are not formed through oxidative mechanisms (23). Here we report that \(\alpha\)TH and its derivatives are effective in preventing UVB induced DNA photodamage. Because DNA damage is a critical event in cancer initiation, inhibition of thymine dimer formation is a relevant parameter for assessing the efficacy of topical chemopreventive agents.

Materials and methods

Abbreviations: \(\alpha\)TH, \(\alpha\)-tocopherol; \(\alpha\)TAc, \(\alpha\)-tocopherol acetate; \(\alpha\)TOMe, \(\alpha\)-tocopherol methyl ether; \(\alpha\)TS, \(\alpha\)-tocopherol succinate; HPLC, high performance liquid chromatography; PABA, 4-aminobenzoic acid; SPF, sun protection factor; UVB, ultraviolet B.

Reagents

R.R.R-\(\alpha\)TH used in these experiments was provided by Henkel Fine Chemicals (LaGrange, IL). d,l-\(\alpha\)TAc was purchased from Sigma Chemical Company (St}

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Louis, MO). α-Tocopherol methyl ether (αTOME) was synthesized from RRR-αTH by Drs Yushun Li and Eugene Mash at the Southwest Environmental Health Sciences Center Synthetic Core Laboratory. αTOME was prepared by potassium hydroxide-catalyzed alkylation of αTH by methyl iodide in dimethylsulfide.

Comparison of UV spectra

A 500 µM solution in methanol of each tocopherol derivative was scanned for absorbance in the UV range using a Beckman 640B spectrophotometer (Beckman Instruments, Fullerton, CA). Although this amount depicted ~44 times less tocopherol than a typically applied 10% dispersion (w/v) contained, this concentration was used to compare the absorbance properties of these compounds within the linear range for Beer’s law.

Topical treatments and UVB irradiation

Female C3H/HeNtac mice (Taconic, Germantown, NY), 10–14 weeks old, were used in all experiments. Four mice per test group were analyzed and results confirmed in duplicate experiments. Test compounds were mixed in a neutral cream vehicle, Vanicream (Pharmaceutical Specialties, Inc., Rochester, MN), in 1%–15% (w/v) dispersions. Commercial SPF rated sunscreen products were applied as prepared by the manufacturer. An aliquot containing 50 mg of each cream was applied to a 3 cm × 4 cm shaved area of the back of each mouse 15 min prior to irradiation. Controls received vehicle only. No significant difference in dimer levels between vehicle treated and untreated controls was detected (not shown).

A bank of six Westinghouse FS20 lamps (National Biological Corp., Twinsburg, OH) was used. Approximately 80% of their output in the UVB, <1% in the UVC, 4% in the UVA, and the remainder in the visible spectrum were used as the UV source. These lamps have been used in numerous photocarcinogenesis and sunscreen studies (18,21,22). Lamps were mounted 19.5 cm above the cage bottom and emitted a dose of 2.5 J/m²/s as measured by a UVP radiometer (UVP, Inc., San Gabriel, CA). Mice were irradiated for 60 min. A dose response study (not shown) determined that this UVB dose induced dimers to a level sufficient to accurately assess inhibition of dimers by the test compounds. This dose is also similar to that used daily in previous photocarcinogenesis studies with tocopherols and their derivatives (21,22).

Dimer inhibition studies also were performed using cutoff filters (Eastman Kodak Co., Rochester, NY) which block wavelengths lower than 285 nm to simulate ‘pure’ UVB. In these studies, increasing amounts of αTH were topically applied as before. Mice were placed in cages which were covered with filters and exposed to FS20 lamps at a dose of 2.5 J/m²/s for 2 h. During irradiation, it is possible that some of the topically applied αTH is photooxidized. Previous experiments show that αTH is rapidly depleted in UVB irradiated liposomes containing αTH (24). The resulting αTH photoproducts may contribute to sunscreening activity since some of these products, including tocopherol quinone, tocopherol epoxides, and a dihydroxy dimer of αTH absorb in or near the UVB.

DNA preparation

After irradiation, mice were killed by CO₂ asphyxiation, their dorsal skin was removed and the epidermal cells were isolated by a previously described method (25). Briefly, skin was placed in 55°C water for 35 s then immersed in ice water for 1.5 min. The epidermal cells were then scraped away from the dermis and frozen immediately at −70°C until DNA extraction could be performed. Epidermal cells were isolated and frozen within 30 min post-UVB exposure. DNA was extracted with the G-NOME kit (Bio 101, Inc., La Jolla, CA). Approximately 50 µg DNA was subjected to hydrolysis in 88% formic acid at 125°C for 20 min. This hydrolysate was evaporated in vacuo and resuspended in 150 µl of distilled water.

Thymine dimer analysis

Thymine dimers were analyzed by a previously described method (26) with modifications. Following hydrolysis, the dimer-containing fraction was separated from unmodified bases by reverse phase HPLC with a Spherisorb ODS-2, 5 µm. 250 × 4.6 mm column, eluted with 100% water at a flow rate of 1.5 ml min⁻¹. The dimer eluted at ~8.5 min and was detected by UV absorbance at 215 nm. This fraction was evaporated and resuspended in 150 µl acetonitrile. Potassium carbonate crystals (15 mg) and 6 µmol pentafluorobenzyl bromide were added and the reaction mixture was heated to 90°C for 6 h. Samples were then evaporated under nitrogen and were resuspended in toluene. Analysis of the pentafluorobenzyl derivative of the dimer was done by capillary gas chromatography with electron capture detection on a Hewlett Packard 5890 Series II gas chromatograph equipped with an HP7673 autosampler (Hewlett Packard, Palo Alto, CA). Samples were introduced by on-column injection and analyzed with a 10 m Hewlett Packard DB-5 column, 0.33 mm i.d. with a 1.5 mm film thickness. The temperature was initially held at 100°C for 1 min and then programmed at 12°C min⁻¹ to 280°C, where it was held for 32 min. The dimer eluted at ~25 min. A calibration curve was constructed with authentic dimer subjected to the same derivatization and analysis protocol. Control experiments (not shown) established that the dimer was stable under the conditions used for DNA hydrolysis and workup. The limit of quantitation was 1 pmol injected dimer.

Dimer levels were normalized to thymine content. An aliquot of the hydrolysate was removed and 26 µg 5-ethyluracil was added as an internal standard. Thymine and 5-ethyluracil were analyzed on a Spherisorb ODS-2, 5 µm, 250 × 4.6 mm column with 5 mM KH₂PO₄, pH 7 and methanol (98.2; v/v) as the mobile phase. Pyrimidines were detected by monitoring UV absorbance at 260 nm. Dimers were expressed as pmol thymine dimer/µmol thymine in the same DNA hydrolysate.

Statistical analysis

Results were analyzed for statistical significance by ANOVA. Treatment groups were compared to control values.

Results

Comparison of UV spectra of tocopherols and derivatives

The absorbance spectra of αTH, αTAc, and αTOME at equimolar concentrations in methanol are depicted in Figure 1. αTAc and αTOME had less total absorbance within the UVB range than did αTH. Their absorbance maxima are at lower wavelengths and their molar absorptivities are lower than for αTH. UV spectra of αTH, γTH, and δTH at equimolar concentrations in methanol are depicted in Figure 2. The absorbance maxima for all three tocopherols lies within the lower UVB range. However, the molar absorptivities of γTH and δTH were higher than for αTH, as were their total absorbances within the UVB range.

Rationale for selection of UV irradiation source and roles of UV wavelengths in dimer formation

Mice were irradiated with Westinghouse FS20 sunlamps, which have been used as UV sources in previous photocarcinogenesis studies involving αTH and its derivatives and sunscreen studies. These lamps cannot be considered ‘pure’ UVB sources. In the description of our experiments, the term ‘UVB’ is an operational designation for the principal wavelength output from the FS20 lamps. Output in the UVA and visible wavelengths from these lamps may contribute to photocarcinogenesis, but not significantly to dimer induction. The small fraction of emission in the upper UVC (~270–290 nm) contributes significantly to dimer yield, as indicated by control experiments in which irradiation through a 285 nm cutoff filter (Eastman Kodak Co., Rochester, NY) reduced dimer yields by ~89% (not shown). Dimer induction thus can be attributed to a combination of UVB and upper UV radiation.

Inhibition of thymine dimer formation by αTH

Mice were irradiated with a UVB dose that, when given chronically, induced skin cancer in mice (21). A 60 min exposure to 2.5 J/m²/s UVB resulted in the formation of 247 ± 42 pmol thymine dimers/µmol thymine. Topical application of αTH in a neutral cream dispersion caused a dose dependent inhibition of thymine dimer formation in epidermal DNA (Figure 3). Significant inhibition (P < 0.05) was observed with dispersions containing 1% or more αTH (w/v). A 1% αTH dispersion reduced dimer formation to 43% of control. The maximum reduction observed was 84% less than controls which was attained at 10% (w/w) αTH.

The UVC portion of the FS20 lamps contributes significantly to dimer induction. Since human exposure to UVC is limited, it was important to confirm that αTH could protect against dimer induction by wavelengths solely in the UVB or higher. Application of αTH and subsequent irradiation through a
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Fig. 1. The UV absorbance spectra of αTH derivatives at a concentration of 500 µM in methanol. Absorbance spectra of αTH (solid line), αTAc (dotted line), and αTOMe (dashed line) and the wavelength limits of the UVB range are depicted.

Fig. 2. The UV absorbance spectra of αTH, γTH and δTH at a concentration of 500 µM in methanol. Absorbance spectra of αTH (dashed line), γTH (solid line), and δTH (dotted line) and the wavelength limits of the UVB range are depicted.

Fig. 3. Inhibition of UVB induced thymine dimers in mouse epidermal DNA by αTH. C3H/HeN mice were treated with increasing concentrations of αTH. They were then exposed to UVB and epidermal DNA was analyzed for thymine cyclobutane dimers as described under Materials and methods. Each bar represents mean ± standard deviation for four animals. Statistically significant differences compared to controls are indicated (*P < 0.05, **P < 0.01).

Fig. 4. Inhibition of thymine dimers induced by filtered UVB sources in mouse epidermal DNA by αTH. C3H/HeN mice were treated with increasing concentrations of αTH. They were then exposed to UVB filtered through 285 nm cutoff filters. Epidermal DNA was analyzed for thymine cyclobutane dimers as described under Materials and methods. Each bar represents mean ± standard deviation for four animals. Statistically significant differences compared to controls are indicated (**P < 0.01).

285 nm cutoff filter demonstrated that αTH inhibits the formation of dimers induced by UV wavelengths higher than 285 nm (Figure 4) in a dose dependent manner. A 1% dispersion (w/w) of αTH reduced dimers to 60% of control and a 10% dispersion significantly reduced dimers to 17% of control (P < 0.01). Since αTH acted similarly as a sunscreen in both filtered and unfiltered irradiations, subsequent irradiations were carried out using unfiltered FS20 sources.

Inhibition of thymine dimer formation by different forms of αTH

Dispersions containing αTAc also produced dose-dependent inhibition of dimer formation, although the effect was smaller than that observed with equivalent amounts of αTH (Figure 5). A 10% (w/w) dispersion of αTAc was necessary to inhibit dimer formation to 44% of controls, which is comparable to the inhibition produced by 1% αTH.

Other forms of vitamin E were similarly studied (Figure 6). αTOMe is spectrally similar to αTAc but is not hydrolyzed to αTH. This allowed us to investigate whether hydrolysis of the acetate ester to form free αTH is necessary for the sunscreens effect. A 10% (w/w) αTOMe dispersion conferred a 50% inhibition of dimer formation (P < 0.05) which is similar to that produced by both 10% αTAc and 1% αTH. Both γTH and δTH were less potent sunscreens than αTH. Dispersions containing 5% (w/w) γTH or δTH significantly (P < 0.05)
Fig. 5. Inhibition of UVB induced thymine dimers in mouse epidermal DNA by αTAc. C3H/HeN mice were treated with increasing concentrations of αTAc. They were then exposed to UVB and epidermal DNA was analyzed for thymine cyclobutane dimers as described under Materials and methods. Each bar represents mean ± standard deviation for four animals. Statistically significant differences compared to controls are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Fig. 6. Inhibition of UVB induced thymine dimers in mouse epidermal DNA by other tocopherol derivatives. C3H/HeN mice were treated with increasing concentrations of tocopherol derivatives. They were then exposed to UVB and epidermal DNA was analyzed for thymine cyclobutane dimers as described under Materials and methods. Each bar represents mean ± standard deviation for four animals. Statistically significant differences compared to controls are indicated (*$P < 0.05$).

Fig. 7. Inhibition of UVB induced thymine dimers in mouse epidermal DNA by commercial sunscreens. C3H/HeN mice were treated with increasing concentrations of PABA or commercial sunscreens with increasing SPF ratings. They were then exposed to UVB and epidermal DNA was analyzed for thymine cyclobutane dimers as described under Materials and methods. Each bar represents mean ± standard deviation for four animals. Statistically significant differences compared to controls are indicated (**$P < 0.01$, ***$P < 0.001$).

reduced dimer formation to 45% of control, which was similar to the level of inhibition produced by 1% αTH.

Inhibition of thymine dimers by PABA and commercial sunscreens

The prototypical suncreening agent PABA also was evaluated. Once widely used, this compound induced skin hypersensitivity reactions and increased DNA photodamage in vitro (27,28) and is no longer used in sunscreen products. Application of PABA resulted in a dose-dependent inhibition of dimers (Figure 7). A 10% (w/w) dispersion was necessary to produce significant ($P < 0.01$) inhibition of dimer formation. This dose reduced dimers to 51% of control, which is similar to the reduction produced by the 1% αTH dispersion.

The sunscreen products used contained proprietary formulations of oxybenzone, octyl methoxycinnamate, octyl salicylate, or PABA esters as active ingredients. Application of a sunscreen containing octyl dimethyl PABA and oxybenzene and rated with a sun protection factor (SPF) 8 provided protection against dimer formation similar to that of the 1% (w/w) αTH dispersion (Figure 7). The SPF 8 sunscreen reduced dimers to 48% of control. However, statistically significant protection was seen only with the SPF 15 ($P < 0.05$) and SPF 30 ($P < 0.01$) preparations, each of which contained octyl methoxycinnamate, octyl salicylate, and oxybenzone.

Discussion

We have demonstrated that topical application of naturally occurring αTH and several of its derivatives can inhibit the formation of thymine dimers in epidermal DNA of UVB irradiated mice. Photodimerization serves as a more specific indicator of UVB energy deposition into DNA than more frequently assessed endpoints, such as edema and erythema. Those endpoints represent complex events resulting from numerous responses elicited by irradiation. Some sunscreens have previously been evaluated for their ability to inhibit the formation of pyrimidine dimers (18). Here we report that αTH and several of its derivatives compare favorably with commercially available sunscreens in their ability to prevent UVB induced DNA photodamage.

Although the lamps used in this study emit light primarily in the UVB, they also emit light in both the UVA and UVC. We have shown that αTH protects against dimer formation induced by both unfiltered sources and by irradiation through a filter that excludes wavelengths lower than 285 nm. As noted in the Results section, however, the contribution of a relatively small amount of UVC to cyclobutane dimer induction is significant. Our experiments with unfiltered FS20 lamps suggest that the photoprotective effect exerted by the compounds tested extends into UVC wavelengths also. Thus, the data obtained in these studies probably provides an accurate indication of the ability of αTH, its derivatives, and sunscreens to inhibit thymine dimer formation induced by any of the UV components of sunlight. Furthermore, the Westinghouse FS20 lamps are widely used in carcinogenesis studies and αTH was shown to prevent photocarcinogenesis induced with these light sources.

Historically, the protective effects of αTH have been attrib-
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uted to its radical scavenging activity. αTH is the primary lipid soluble chain breaking antioxidant in cells (29). Consequently, αTH or its more thermally stable derivative, αTAc, is often added to skin care products as an antioxidant supplement. However, the direct sunscreening effect of αTH demonstrated here is a striking addition to the growing list of effects αTH has on general cellular function. αTH and certain tocopherol derivatives can modify signal transduction pathways (30,31), improve immune responsiveness (32,33), inhibit platelet phospholipase A2 (34) and suppress proliferation of certain cell types in vitro (35–37). All of these actions may contribute to their antitumorigenic and photoprotective potential. Our results suggest, however, that prevention of DNA photodamage may account for much of the antitumor effect of topically applied αTH in photocarcinogenesis.

Furthermore, prevention of DNA photodamage may reflect reduced incident UVB penetration into the epidermis and dermis. Therefore, the photoprotective effect of αTH may extend to prevent other types of DNA photodamage and of damage to biomolecules involved in UVB-associated responses. It is reasonable that αTH could inhibit the formation of pyrimidine (6–4) pyrimidone photoproduets, since they form through similar UV induced photochemistry, and perhaps DNA photolyases. Furthermore, topical application of αTH has been shown to reduce UVB induced immunosuppression (21). Photoprotection also may involve prevention of acute responses including activation of growth related genes such as c-fos (38) and c-H-ras (39). This could prevent early alterations in cellular metabolism that promote tumor formation. Manifestations of chronic UVB exposure, such as solar elastosis and dermal degeneration (40) also could be attenuated.

αTH may actually be a more effective DNA photoprotectant than the active ingredients in sunscreen preparations. A dispersion containing 1% αTH was as potent as products containing multiple sunscreensing agents. Thus, a particular combination or higher concentrations of these sunscreensing agents may be necessary to reach the same level of protection demonstrated by αTH. Further investigations of αTH and specific sunscreen components are underway in our laboratory to examine this issue.

Despite the structural similarity between the forms of vitamin E tested, αTH is a more potent inhibitor of DNA photodamage than the other tocopherols examined. Since αTAc may be hydrolyzed in cells to form αTH, the sunscreensing effect exerted by αTAc could depend in part on its hydrolysis to αTH. Experiments with the non-hydrolyzable tocopherol derivative αTOMe indicated, however, that hydrolysis may not be necessary. Equal concentrations of αTOMe and αTAc, which have nearly identical spectral properties, produced a similar degree of inhibition of dimer formation. Thus, hydrolysis to αTH may not contribute significantly to the photoprotective effect of αTAc when acutely applied.

It is particularly interesting that αTH is ~five times more potent than γTH and δTH. This is surprising since both γTH and δTH have slightly greater absorbances within the UVB range than αTH. However, bioassay comparisons of the various tocopherols indicated that αTH had the highest biological vitamin E activity (41,42). Binding proteins specific for αTH also have been isolated from membrane fractions of human erythrocytes (43), from the cytosol of rat liver and heart (44), from bovine aortic endothelial cells (45), from bovine (46) and rabbit heart cytosol (47) and human liver (48). These purified proteins bind αTH in preference to δTH and γTH.

Thus, the αTH form may be preferentially absorbed by cells and more effectively distributed to critical intracellular targets.

Our findings emphasize the need to ascertain which forms of tocopherol are active photoprotectants. Although αTH is a superior sunscreen and antioxidant, the thermally stable αTAc derivative is more commonly used in sunscreen preparations and other skin care products. However, recent work by Gensler et al. (22) indicated that whereas topically applied αTH prevented UVB photocarcinogenesis, neither αTAc nor αTS did so under the same conditions. Our results provide a plausible mechanistic explanation for these differences.

Our studies indicate a novel mechanism by which αTH can inhibit photocarcinogenesis. The ability to attenuate DNA damage could prevent mutations in critical genes associated with photocarcinogenesis. Thus, the sunscreensing activity of αTH may be as important as antioxidant activity in preventing cellular damage and tumor formation. Further studies will be needed to explain why αTH exhibits superior sunscreensing actions over other tocopherols and commercial sunscreens in vivo.

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