See related commentary on page 1448

ORIGINAL ARTICLE

Topical Application of a Novel, Hydrophilic γ-Tocopherol Derivative Reduces Photo-Inflammation in Mice Skin

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We previously demonstrated that a novel hydrophilic γ-tocopherol (γ-Toc) derivative, γ-tocopherol-*N*, *N*-dimethylglycinate hydrochloride (γ-TDMG) converts to γ-Toc in the mouse skin and has a higher bioavailability than γ-Toc itself. In the present study, we determined whether γ-TDMG could reduce photo-inflammation in mouse skin, and compared its effectiveness to that of α-Toc acetate (α-TA). Topical pre- or post-application of 5% γ-TDMG significantly reduced the formation of edema and tempered the increase in cyclooxygenase-2 (COX-2)-catalyzed synthesis of prostaglandin E_2 (PGE_2) that were induced by a single dose of UV irradiation of 2 kJ/m^2 (290–380 nm, maximum 312 nm). The pre-treatment of mouse skin with 10% α-TA had the same anti-inflammatory effect as did γ-TDMG. In spite of same having the ability to reduce PGE_2 levels, the effect of γ-TDMG pre-treatment on the inhibition of COX-2 mRNA/protein expression was less than that seen with 10% α-TA. In contrast, the increase in COX-2 activity seen after UV exposure was reduced more by γ-TDMG than by α-TA, suggesting that the reduction in PGE_2 levels might have been due to the direct inhibition of COX-2 activity by γ-TDMG-derived γ-Toc. Both Toc derivatives strongly suppressed inducible nitric oxide synthase (iNOS) mRNA expression and nitric oxide (NO) production, both of which play important roles in UV-induced inflammation. Both derivatives also significantly reduced lipid peroxidation in response to UV exposure, though γ-TDMG's ability in this regard was less than that seen with α-TA, which correlated with their abilities to suppress COX-2 expression. Thus, the γ-TDMG-derived γ-Toc acts as an antioxidant, suppresses iNOS expression and directly inhibits COX-2 activity, all of which likely play a role in mediating its suppressive effects on photo-inflammation. Our data further suggest that the topical application of γ-TDMG, a novel hydrophilic γ-Toc derivative, may be efficacious in preventing and reducing UV-induced inflammation in humans.

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INTRODUCTION

Exposure of the skin to UV radiation induces a variety of biologic effects including inflammation, characterized by immunosuppression and erythema/edema (Hruza and Pentland, 1993), the latter of which is primarily mediated by prostaglandin E_2 (PGE_2) and is the most prominent visible sign of UV-induced inflammation in human skin (Miller *et al.*, 1994; Buckman *et al.*, 1998; Wilgus *et al.*, 2000; Wilgus *et al.*, 2002). Increased production of PGE_2 in response to UV exposure was reported to be due to enhanced expression of cyclooxygenase-2 (COX-2) in the epidermis (Hla and Neilson, 1992; Grewe *et al.*, 1993; Pentland, 1994; Buckman et al., 1998; Isoherranen *et al.*, 1999). The importance of nitric oxide (NO) as a mediator of skin inflammation was confirmed by the demonstration that UVB exposure increased the expression of inducible nitric oxide synthase (iNOS) mRNA and NO itself in human keratinocytes (Chang *et al.*, 2003). Both NO and PGE_2 were shown to play roles as secondary messengers in the inflammatory signaling cascade (Notoya *et al.*, 2000).

Tocopherol, a class of fat-soluble phenolic compounds with varying degrees of vitamin E antioxidant activity, exists as four homologous, that is, α, β, γ, and δ, which differ in the number and position of methyl groups in their chroman ring. It is generally been assumed that α-tocopherol (α-Toc) is the most biologically active lipophilic antioxidant in many biological systems (Traber and Sies, 1996). Topical application of α-Toc was shown to inhibit the proliferation of UVB-induced tumors (Gensler and Magdaleno, 1991), edema, erythema, and lipid peroxidation (Trevithick *et al.*, 1992);

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Abbreviations: α-Toc, α-tocopherol; α-TA, α-Toc acetate; γ-CEHC, γ-carboxyethyl-hydroxychromans; γ-TDMG, γ-tocopherol-N,N-dimethylglycinate hydrochloride; γ-Toc, γ-tocopherol; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PGE_2, prostaglandin E_2; ROS, reactive oxygen species

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it was also reported to block UV-induced oxidative skin damage. It has been suggested that γ-tocopherol (γ-Toc) might have more than just antioxidant effects. Specifically, γ-Toc was found to be superior to α-Toc in its ability to scavenge nitrogen oxides, which are mutagenic electrophiles that are generated during inflammation (Cooney *et al.*, 1993; Cooney *et al.*, 1995; Christen *et al.*, 1997; Hoglen *et al.*, 1997). γ-Toc is efficiently metabolized to γ-carboxyethyl-hydroxychromans (γ-CEHC), the latter of which was shown to exhibit natriuretic activity (Wechter *et al.*, 1996; Kantoci *et al.*, 1997; Murray *et al.*, 1997). γ-Toc and its major metabolite were recently shown to reduce PGE_2 levels *in vitro* (Jiang *et al.*, 2000) and *in vivo* (Jiang *et al.*, 2002; Jiang and Ames, 2003), suggesting that it acts as an anti-inflammatory agent.

γ-Toc is nearly insoluble in water and is readily oxidized by atmospheric oxygen. The phenolic functional group in γ-Toc is readily esterified and some of these ester derivatives were shown to have improved water solubility and resistance to oxidation. γ-tocopheryl-dimethyl-glycinate (γ-TDMG), the *N,N*-dimethylglycine ester of γ-Toc, was found to be water soluble and hydrolyzable by esterases in rat and human liver microsomes (Takata *et al.*, 2002). We previously demonstrated that topically applied γ-TDMG suppressed UV-induced erythema/edema formation in mouse skin and displayed more tissue availability than γ-Toc (Yasuoka *et al.*, 2005).

In this study, we demonstrated that topically applied γ-TDMG could suppress UV-induced inflammation in skin and also examined its mechanism of action.

RESULTS

Changes of the concentrations of cutaneous γ- and α-Toc after 5% γ-TDMG- or 10% α-Toc acetate (α-TA)-treatment, respectively, in mice

A 5% solution of γ-TDMG or 10% α-TA was topically applied and left on mouse skin for 1 hour, after which the solutions were removed with 70% ethanol. Our results showed that such treatment increased cutaneous γ-Toc levels by approximately 32 μM after 1 hour, which represented a 15-fold increase in endogenous levels, and reached 80 μM 24 hours later (Table 1). α-Toc levels rose to similar levels (92 μM) when 10% α-TA was used. The fact that 5% γ-TDMG- and 10% α-TA-treated skins contained the same concentrations of γ-Toc and α-Toc, respectively, validated our assessment of the effects of these compounds on parameters of inflammation.

Protective effects of 5% γ-TDMG and 10% α-TA on UV-induced edema/inflammation

Dorsal skin samples that were exposed to UV irradiation displayed a 1.5-fold increase in thickness compared to non-exposed skin after 24 hours. Pretreatment of the skin with 5% γ-TDMG or 10% α-TA significantly suppressed this UV-induced edema (Figure 1). In post-treated skin, γ-TDMG similarly had the ability to suppress edema though α-TA did not.

Table 1. Levels of cutaneous α- and γ-Toc in mice after α-TA- or γ-TDMG treatment

	(nmol/g wet weight)		
	Normal	**5% γ-TDMG**	**10% α-TA**
γ-Toc after			
0 hour	1.9±0.3	32.0±4.9	
24 hours		80.8±13.8	
α-Toc after			
0 hour	6.3±0.7		32.9±6.8
24 hours			92.4±16.8

Abbreviations: γ-TDMG: γ-tocopherol-*N,N*-dimethylglycinate hydrochloride; γ-Toc: γ-tocopherol; α-Toc: α-tocopherol.
A 5% solution of γ-TDMG or 10% α-TA was topically applied to the dorsal skin of mice and left on for 1 hour, after which the solutions were removed with 70% ethanol. After 0 hour and 24 hours, the concentrations of γ-Toc and α-Toc were determined by HPLC. Each number represents the means±SE of six animals.

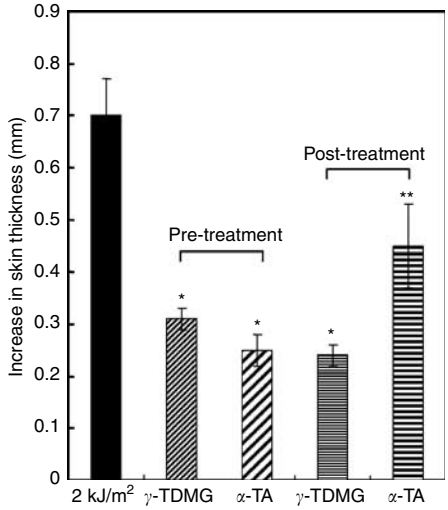

Figure 1. Effects of topically applied γ-TDMG and α-TA on edema/inflammation induced by UV irradiation. γ-TDMG (5%) and α-TA (10%) were topically applied to and left on, the dorsal skin for 1 hour before (pretreatment) or after (post-treatment) UV-exposure (2 kJ/m^2). The skin samples were removed and their thickness measured 48 hours later. Each bar represents the mean±SE of 12 skin samples from six mice. *$P<0.01$ relative to irradiated; **$P<0.05$ relative to irradiated.

Inhibition of UV-induced production of PGE_2 by tocopherol derivatives

In light of the suppression of UV-induced edema by γ-TDMG, we measured the amount of PGE_2, a primary mediator of inflammation, in UV-exposed mouse dorsal skin after 24 hours by enzyme immunoassay. PGE_2 levels in the exposed skin were approximately twice (145±5 pg/mg protein) those seen in non-exposed skin (82±9 pg/mg protein). As shown in Figure 2, this increase in UV-induced PGE_2 levels was significantly reduced by pretreatment with γ-TDMG, α-TA, or indomethacin. Post-treatment with γ-TDMG, but not α-TA, also significantly reduced PGE_2 levels.

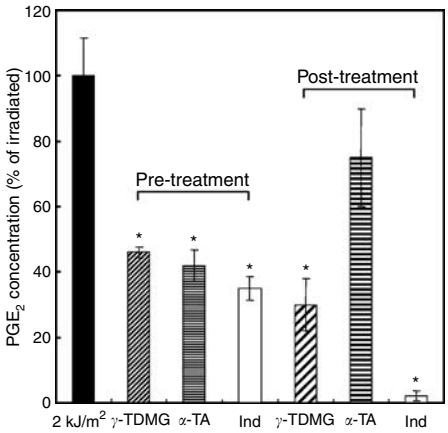

Figure 2. Inhibition of UV-induced PGE_2 production by tocopherol derivatives. Effects of pre- and post-treatment with 5% γ-TDMG, 10% α-TA, or 5% indomethacin on PGE_2 levels in skin 24 hours after UV exposure. PGE_2 levels are expressed as a percentage of its concentration in irradiated, non-treated skin. Each bar represents the mean ± SE of 12 skin samples from six mice. *$P<0.01$ relative to irradiated.

Effects of γ-TDMG and α-TA on UV-induced COX-2 mRNA/protein expression

Since COX-2 catalyzes the synthesis of PGE_2, we measured its mRNA/protein expression by RT-PCR assay and Western blotting, respectively. The expressions of COX-2 mRNA (Figure 3a) and protein (Figure 3b) in UV-exposed skin were approximately 10-fold greater than in non-exposed skin. Pretreatments of the skin with γ-TDMG and α-TA significantly suppressed COX-2 mRNA/protein expression by 30 and 50% of the irradiated control, respectively. Although γ-TDMG had the same ability to reduce PGE_2 levels as did α-TA (Figure 2), it was less effective in inhibiting COX-2 expression (Figures 3a and b).

Effects of tocopherol derivatives on COX-2 enzyme activity

COX-2 activity increased by 5-fold in irradiated skin (Figure 4). This increase was significantly reduced by 60, 40, and 85% in samples that were pretreated with γ-TDMG, α-TA, or indomethacin, respectively; thus, γ-TDMG was more effective than α-TA at inhibiting COX-2 activity, in spite of being less effective in inhibiting COX-2 gene/protein expression.

Effects of tocopherol derivatives on iNOS mRNA expression and generation of nitrite in UV-exposed skin

Because NO acts as a messenger in the inflammatory cascade, 24 hours after UV irradiation, iNOS mRNA expression and NO levels were determined in the mouse skin samples. UV exposure led to a marked increases in iNOS mRNA levels (Figure 5a); γ-TDMG and α-TA strongly suppressed this expression. The generation of NO was indirectly measured by quantifying nitrite formation (Figure 5b). NO production in UV-exposed skin was approximately four times that in non-exposed skin, and its production was significantly reduced by pretreatment with γ-TDMG or α-TA.

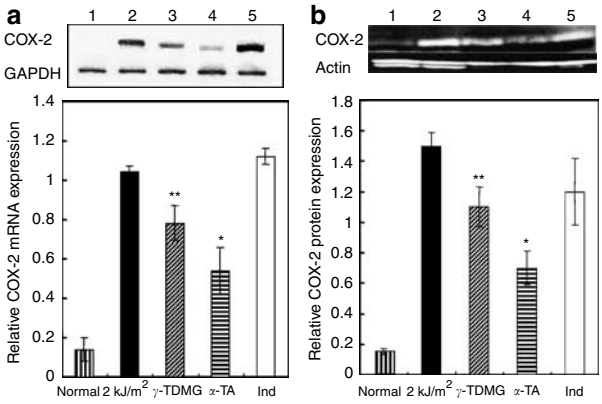

Figure 3. Effect of tocopherol derivatives on UV-induced COX-2 expression. (**a**) Skin samples were removed 8 hours after UV exposure. Total RNA was extracted from skin samples that were the treated with γ-TDMG or α-TA for 1 hour and then irradiated. COX-2 mRNA levels were analyzed by RT-PCR method using a specific COX-2 mRNA primer, and mRNA levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1, normal; lane2, irradiated; lane 3, 5% γ-TDMG treated, irradiated; lane 4, 10% α-TA treated, irradiated; lane 5, 5% indomethacin treated, irradiated. The bars represent the relative levels of the COX-2 PCR band compared to GAPDH; values were obtained using a densitometer. (**b**) The skin samples (200 mg wet weight) that were treated with Toc derivatives were removed 24 hours after UV exposure, frozen and then crushed in a mortar on dry ice. The lysates (40 μg protein per lane) were used for Western blotting. Lane 1, normal; lane2, irradiated; lane 3, 5% γ-TDMG treated, irradiated; lane 4, 10% α-TA treated, irradiated; lane 5, 5% indomethacin treated, irradiated. The bars represent the relative levels of the COX-2 blotting band compared to β-actin; values were obtained using a densitometer. Each bar represents the mean ± SE of three experiments. *$P<0.01$ relative to irradiated; **$P<0.05$ relative to irradiated.

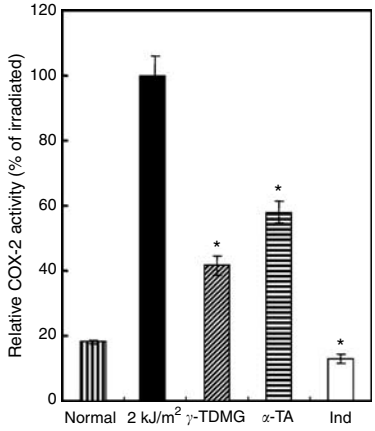

Figure 4. Inhibition of COX-2 activity by γ-TDMG or α-TA treatment. (**a**) γ-TDMG, α-TA, and indomethacin pretreated skins were frozen in liquid nitrogen, crushed and homogenized in 100 mM Tris-HCl buffer, pH7.5, 24 hours after being irradiated. After centrifugation, the supernatants were assayed using a COX-2 assay kit as described in Materials and Methods. Each bar represents the mean ± SE of 12 skin samples from six mice. *$P<0.01$ relative to irradiated.

Protective effects of γ-TDMG, α-TA, and indomethacin on UV-induced lipid peroxidation

The concentration of cutaneous thiobarbituric acid reactive substance in irradiated skin was twice (26.25 ± 5.6 nmol/mg

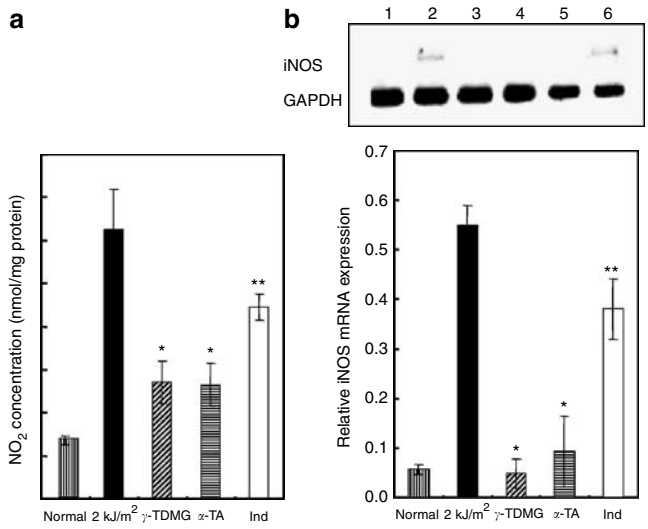

Figure 5. Effects of γ-TDMG, α-TA, and indomethacin on UV-induced iNOS mRNA expression and nitrite production. (**a**) Total RNA was extracted from the γ-TDMG, α-TA, and indomethacin treated, irradiated skins. iNOS mRNA levels were analyzed using RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1, normal; lane 2, irradiated; lanes 3 and 4, 5% γ-TDMG treated, irradiated; lane 5, 10% α-TA treated, irradiated; lane 6, 5% indomethacin treated, irradiated. The bars represent the relative levels of iNOS compared to GAPDH, as determined by densitometry. (**b**) γ-TDMG-, α-TA-, and indomethacin-treated, irradiated skins were homogenized and centrifuged, and their nitrite concentration determined in their supernatants using the fluorometric DAN test kit. Each bar represents the mean ± SE of three experiments. *$P<0.01$ relative to irradiated, **$P<0.05$ relative to irradiated.

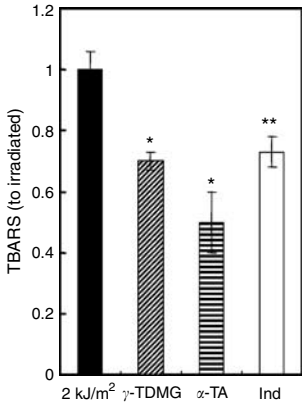

Figure 6. Effects of topically applied γ-TDMG and α-TA on lipid peroxidation induced by UV-exposure. Each derivative as well as indomethacin was topically applied to the dorsal skin 1 hour before UV exposure; the skin samples were then assayed 24 hours later. Each bar represents the mean ± SE of three experiments. *$P<0.01$ relative to irradiated, **$P<0.05$ relative to irradiated.

protein) that found in non-irradiated skin (12.8 ± 2.7 nmol/mg protein). This increase was significantly inhibited by pretreatment with γ-TDMG, α-TA, or indomethacin. The relative inhibition by these substances was α-TA > γ-TDMG > indomethacin; thus, α-TA appeared to be more effective than γ-TDMG in scavenging reactive oxygen species (ROS) (Figure 6).

DISCUSSION

Our data showed that the topical application of the novel, hydrophilic γ-Toc derivative, 5% γ-TDMG before or after irradiation significantly prevented edema/inflammation induced by exposure to a single dose of UV irradiation. Post-treatment with γ-TDMG was also effective. While α-TA also afforded protection when it was applied before irradiation, it was not effective when given after irradiation. These results suggest that the anti-inflammatory effects of γ-TDMG might not be wholly attributable to its ability to scavenge UV-generated ROS or to have a sunscreening effect.

It was recently reported that PGE_2 plays a role in modulating signal transduction in the inflammatory pathway. The UV exposure of skin has been shown to induce the synthesis of PGs (Grewe *et al.*, 1993; Buckman *et al.*, 1998). We demonstrated that both pre- and post-treatments with γ-TDMG significantly suppressed UV-induced PGE_2 production, supporting the notion that the suppression of UV-induced inflammation by our test compounds was due to the suppression of PGE_2. COX-2 protein levels increased after UVB exposure in the skins of both humans (Buckman *et al.*, 1998) and mice (Athar *et al.*, 2001). Since COX-2 is one of the key enzymes required for the synthesis of PGE_2 from arachidonic acid (Smith *et al.*, 1996), we investigated the effects of γ-TDMG on COX-2 mRNA/protein expression in irradiated skin. In spite of their similar abilities to suppress PGE_2 levels and the greater inhibition of COX-2 activity by γ-TDMG, the latter was less effective in suppressing COX-2 expression than α-TA. It is thought that direct inhibition of COX-2 activity plays a role in mediating the anti-inflammatory effects of γ-TDMG-derived γ-Toc. Jiang *et al.* (2000) found that γ-Toc and its major metabolite, γ-CEHC, suppressed PGE_2 synthesis in both lipopolysaccharide-stimulated macrophages and IL-1β-treated human epithelial cells; our data support their findings. When 5% γ-TDMG was applied to mouse skin for 1 hour, cutaneous γ-Toc levels increased by 30-fold, though γ-CEHC levels remained unchanged (data not shown). Furthermore, γ-CEHC levels did not correlate with COX-2 inhibition in mouse skin. It is theorized that γ-TDMG-derived γ-Toc acts as an antioxidant and inhibitor of COX-2 activity in skin. However, it is not clear whether γ-Toc derivative in itself, or γ-TDMG is responsible for mediating this effect.

NO is known to play a major role in UVA- (Suschek *et al.*, 2001) and UVB- (Suschek *et al.*, 2004) induced inflammation in human skin. NO production was reported to correlate with the expression of iNOS mRNA in keratinocytes (Seo *et al.*, 2002). Jiang *et al.* (2000) reported that γ-Toc and α-Toc suppressed iNOS mRNA expression in lipopolysaccharide-treated macrophages. Moreover, they demonstrated that high doses (100 mg/kg) of γ-Toc, but not α-Toc, reduced total nitrite and nitrate levels in a rat inflammation model (Jiang and Ames, 2003). We showed that both Toc derivatives, that is, γ-TDMG and α-TA, significantly inhibited the UV-induced iNOS mRNA expression and NO production in mouse skin. While several studies suggested the presence of a link between the iNOS and COX pathways, the data in the

literature have been conflicting (Di Rosa *et al.*, 1996; Salvemini, 1997; Clancy *et al.*, 2000). Stimulation of a wide variety of cell types with cytokines upregulates their COX-2 expression, which is often accompanied by an increase in iNOS levels (Vane *et al.*, 1994; Akarasereenont *et al.*, 1995). Since γ-TDMG did not have a strong effect on COX-2 expression, it is likely that γ-TDMG-derived γ-Toc was responsible for inhibiting iNOS expression. Thus, γ-TDMG-derived γ-Toc may act as an anti-inflammatory agent by directly inhibiting COX-2 activity, suppressing iNOS expression and scavenging ROS generated by UV exposure.

It is known that UV irradiation increases cellular ROS levels, resulting in damage to lipids, protein and DNA (Scharffetter-Kochanek *et al.*, 1997; Podda *et al.*, 1998). It is generally assumed that α-Toc is the most biological active lipophilic antioxidant in many biological systems (Traber and Sies, 1996). In our experiment, although both derivatives-treated skins contained similar amounts of Toc, the α-TA more prevented lipid peroxidation (50% of irradiated control) more than did γ-TDMG (30% of irradiated control). Thus, α-TDMG-derived α-Toc probably plays an important ROS scavenging role in response to UV exposure, suggesting that this may have been the mechanism by which α-Toc reduced photo-inflammation.

Our data showed that γ-TDMG was more effective than α-TA in preventing photo-inflammation, despite the fact that both of these compounds led to the accumulation of the same levels of intracellular Toc. We previously reported (Yasuoka *et al.*, 2005) that the topical application of 5% γ-TDMG significantly prevented such parameters of UV-induced mouse skin damage as sunburn cell formation, lipid peroxidation and edema compared to α-TA. Since γ-Toc acts as both an antioxidant and an inhibitor of COX-2, and iNOS expression, it stands to reason that γ-TDMG might be a more effective antiphoto-inflammation agent than α-TA. This contention is supported by the fact that γ-TDMG increased skin γ-Toc levels seven times more than did γ-Toc itself even after 24 hours after administration (Yasuoka *et al.*, 2005). When applied either before or after irradiation, γ-TDMG significantly prevented inflammation. It remains to be seen whether γ-TDMG is more effective in preventing photo-inflammation than non-steroidal anti-inflammatory drugs such as aspirin or indomethacin.

In conclusions, our data suggest that the topical application of γ-TDMG, a novel hydrophilic derivative of γ-Toc, significantly inhibits increases of PGE_2 and NO production, as well as inflammation. In this regard, the effectiveness of γ-TDMG was greater than that of α-TA.

MATERIALS AND METHODS

Chemicals

γ-TDMG was kindly provided by the Showa Denko Company (Tokyo, Japan). δ-Toc and α-TA were purchased from Eizai Co. Ltd. (Tokyo, Japan) and Sigma-Aldrich Fine Chemicals (St Louis, MO), respectively.

Animals

Female, hairless mice (5 weeks old) (SKH-hr1; Sankyo Laboservice Inc., Tokyo, Japan) were used in this study. The mice were housed under standard conditions (fluorescent light 12 hours/day with a room temperature of 23°C and a relative humidity of 45–55%) and were fed a commercial diet and water *ad libitum*. The protocols for all of the animal experiments were approved by the Committee for the Ethical Use of Animals in Experiments at the Kyoritsu University of Pharmacy.

Topical application of the tocopherol derivatives and UV irradiation

The mice were divided into five groups of six mice each. Group 1 was left untreated; group 2 was not treated with derivatives, but was irradiated; groups 3–5 were treated with test compounds and were irradiated mice. Results were confirmed in duplicate experiments. The test compounds, that is, γ-TDMG and indomethacin were separately dissolved in a water:propylene glycol:ethanol solution (2:1:2) to make a 5% solution. α-Toc was dissolved in the same diluents to make a 10% solution. Each of these solutions (60 μl) were applied to a $2 \times 4\,cm^2$ patch of dorsal skin; mice were irradiated either 1 hour after or before application. Control mice received vehicle alone. The area of skin to which Toc derivatives were applied was wiped with 70% alcohol before or 1 hour after. Skin was exposed to a irradiation dose of $2\,kJ/m^2$ from directly above at a distance of 50 cm using a UV lamp (290–380 nm, maximum 312 nm, ATTO Co., Tokyo, Japan); UV doses were determined with an Atto Radiometer (ATR, Atto Co., Tokyo, Japan) that had a maximum sensitivity of 312 nm. Approximately 64% of the radiation was emitted in the range of 291–320 nm, with the rest being emitted in the 321–380 nm range. Exposure time was 30 minutes ($2\,kJ/m^2$), and no heat was detectable during irradiation. Animals were irradiated while housed in stainless-steel cages that were $20 \times 15 \times 4.5\,cm^3$ (six mice/cage). Irradiated skins were collected 24–48 hours after exposure.

Skin thickness

Skin samples from six animals in each group that were treated with tocopherol derivatives, were removed 48 hours after UV exposure. The thickness of the skin was measured at three midline sites using a caliper (Digimatic Micrometer 543; Mitutoyo Co., Tokyo, Japan) that was accurate to 0.01 mm.

Determination of γ-Toc, α-Toc, and γ-CEHC concentrations in skin

Skin samples (100 mg wet weight) were homogenized at 4°C in 1 ml of 200 mM Tris-HCl buffer (pH 7.0). using a Polytron system (Kinematica CH-1600, Littau-Lucerne, Switzerland). α-Toc and γ-Toc, and their derivatives were analyzed using a previously described HPLC method (Nakayama *et al.*, 2003), with some modifications. The analyses were performed using an HPLC system SCL-10A (Shimadzu Co., Kyoto, Japan) in combination with UV/VIS (SPD-10A) and fluorescence (RF-535) HPLC monitors (Shimadzu Co., Kyoto, Japan). The fluorescence absorbance for α-Toc and γ-Toc was monitored at an excitation wavelength of 296 nm and an emission wavelength of 323 nm; the UV absorbance of their derivatives was monitored at a wavelength of 285 nm.

γ-CEHC in γ-TDMG concentrations were determined by HPLC as previously described (Hattori *et al.*, 2001). γ-CEHC in skin homogenates was derivatized with a fluorescent reagent, 4-*N*, *N*-dimethylaminosulfonyl-7-piperazino-2,1,3-benzoxadiazole, and

acetylated with acetyl chloride after deproteinization with adding CH_3CN-EtOH (4:1, V/V). Following purification using an Empore-TMC_{18} cartridge, the sample was injected into the column switching HPLC system. Detection was carried out fluorometrically at 560 nm after excitation at a 450 nm wavelength.

PGE_2 assay

Skin samples (150 mg wet weight) were removed 24 hours after UV exposure and frozen in liquid nitrogen, after which they were crushed in a mortar and then homogenized on dry ice in 4 ml of buffer containing 50 mM Tris-HCl and 0.25% sucrose (pH 8.3) using a Dounce homogenizer; the samples were then centrifuged at $8,000 \times g$, at 4°C for 20 minutes. The supernatants were again centrifuged at 100,000 *g* for 20 minutes at 4°C and after which the pellets were dissolved in 200 μl of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenol. PGE_2 concentrations were measured in the dissolved pellets by a PGE2 ELISA kit from Cayman Chemicals (MI) according to the manufacture's instructions.

COX-2 activity assay

Our COX activity assay was based on the measurement of the peroxidase component of cyclooxygenase. Peroxidase activity was assessed colorimetrically by quantifying the levels of oxidized *N,N,N′,N′*-tetramethyl-*p*-phenylenediamineat 590 nm. Hydroperoxidase activity was determined by spectrophotometry. Skin samples (400 mg wet weight) were prepared as described above for the PGE_2 and 50 μl of supernatants from the centrifuged homogenates ($8,000 \times g$ for 20 minutes) were added into the reaction mixture (COX-1 assay kit, Cayman Chemical; MI) that contained 100 μl of assay buffer, 10 μl of heme, 20 μl of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and inhibitory test compounds. Reactions were initiated by the addition of 20 μl of arachidonic acid to the mixture after which changes in absorbance at 590 nm were measured. Inhibitory activity was calculated by comparing the initial rate of change in absorbance in the presence of test compounds with that observed with the potent COX-2 inhibitor DuP-697 (5-bromo-[4-fluorophenyl]-3-[4-(methylsulfonyl)phenyl] thiophene.

Nitrite determination

Skin samples (100–150 mg wet weight) were minced and homogenized in 10 volumes of 50 mM phosphate buffer, pH 7.8 at 2°C, using a Polytron homogenizer (Kinematica CH-1600, Littau-Lucerne, Switzerland), after which the homogenates were centrifuged at $8,000 \times g$ for 20 minutes at 4°C. The nitrite concentration in the supernatants (80 μl) was measured as an indicator of NO production using a fluorometric DAN (2,3-diaminonaphtalen) test kit (NO_2 Assay Kit-F II, Dojindo Laboratories, Kumamoto, Japan) using $NaNO_2$ as the standard (Marzinzig *et al.*, 1997).

RT-PCR analysis

Skin samples (100 mg wet weight) were removed 8 hours after UV exposure, frozen and then crushed in a mortar on dry ice. Total RNA was isolated from the crushed tissues by using guanidinium isothocyanate and acid phenol/chloroform (Chomczynski and Sacchi, 1987). Single-stranded cDNA was synthesized using a first-strand cDNA synthesis kit (ReverTra Ace-α-^{TM}, TOYOBO Co. Ltd, Osaka, Japan). PCR was performed in 5 μl of PCR buffer, 1 μl of dNTP mixture, 4 μl of sterilized distilled water, 1 μl of primer, and 0.25 μl of Takara Ex Taq^{TM} (Takara Bio Inc., Otsu, Japan). Amplification was initiated at 96°C for 2 minutes, and subsequently run for 60 seconds at 96°C, 60 seconds at 63°C, 120 seconds at 72°C for 25 cycles, followed by a final extension for 7 minutes at 72°C. Amplification of the iNOS gene was run for 35 cycles. The following oligo-nucleotide primers were used: COX-2 (277 bp), 5′-GCATTCT TTGCCCAGCACTT-3′ (forward primer), 5′-AGACCAGGCACCAGA CCAAAGA-3′(reverse primer); iNOS (492 bp), 5′-CAGTGCCCTGCT TTGTGCGAAGT-3′(forward primer), 5′-AACGTTTCTGGCTCTTGA GCTGGAA-3′(reverse primer); glyceraldehyde-3-phosphate dehydrogenase (262 bp), 5′-AATGTATCCGTTGTGGATCT-3′(forward primer), 5′-TCCACCACCCTGTTGCTGTA-3′(reverse primer). PCR products were analyzed by 2% agarose gel electrophoresis. The size of the products was identical to that predicted from the sequences. After staining with ethidium bromide, the gels were viewed under UV light, and digital images were captured with a CCD camera system.

Immunoblot analysis

Skin samples (200 mg wet weight) were removed 24 hours after UV exposure, frozen and then crushed in a mortar on dry ice and then homogenized on dry ice in 4 ml of buffer containing 50 mM Tris-HCl and 0.25% sucrose (pH 8.3) using a Polytron homogenizer; the homogenate was then centrifuged at $8,000 \times g$, at 4°C for 20 minutes. Supernatants were extracted with a lysis buffer containing of 0.25 M Tris-HCl (pH 7.5), 0.375 M NaCl, 1.12% diethyldithiocarbonate, 0.02% phenylmethyl sulfonyl fluoride, and 1% Tween 20. The lysates (~40 μg protein) were separated in 7% polyacrylamide gel by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% nonfat-dried milk in tris-buffered saline-T buffer consisting 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.05% Tween 20, after which they were incubated with anti-COX-2 (murine, polyclonal antibody, Cayman Chemical; 1:1500) or anti-actin (Cayman Chemical; 1:3000) antibodies for 40 hours at 4°C. After several washes with water and tris-buffered saline-T, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (British BioCell International Ltd, UK; 1:3000) for 3–4 hours at room temperature. Following several washes with water and tris-buffered saline-T at pH 9.5, the blots were incubated with CDP-Star chemiluminescent substrate (New England Biolabs, UK). Chemiluminescent signal was detected with FP-3000B instant film (Fujifilm, Tokyo, Japan) using ECL minicamera (GE Healthcare Bio-Sciences Co., NJ).

Lipid peroxidation

Skin samples were homogenized in 10 volumes of 50 mM phosphate buffer, pH 7.8 at 2°C, using a Polytron homogenizer. Lipid peroxidation was measured in these homogenates using a thiobarbituric acid reactive substances assay (Kikukawa *et al.*, 1992). The concentration of thiobarbituric acid reactive substance was expressed per mg protein, the latter of which was determined using the Bradford method with bovine plasma globulin as the standard.

Statistics

Data are expressed as the mean ± SE. Differences between groups were assessed using a one-way analysis of variance followed by Bonferroni-corrected *t*-tests. Differences of $P < 0.05$ were considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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