Diet-derived and topically applied tocotrienols accumulate in skin and protect the tissue against ultraviolet light-induced oxidative stress

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To evaluate the tissue-specific distribution of lipophilic antioxidants including various vitamin E forms (tocotrienols and tocopherols) and oxidised and reduced coenzyme Q (ubiquinone and ubiquinol), a sensitive procedure was developed using gradient HPLC with both electrochemical- and UV-detection. A unique distribution of these antioxidants in hairless mouse tissues was found, suggesting that their distribution may be dependent upon selective mechanisms for maintaining antioxidant defences. Ubiquinol-9 was highest in kidney (81 ± 29 nmol/g) and in liver (42 ± 16 nmol/g), while the highest ubiquinone-9 concentrations were found in kidney (301 ± 123 nmol/g) and heart (244 ± 22 nmol/g). Liver contained nearly identical amounts of each ubiquinol-9 (41 ± 16 nmol/g) and ubiquinone-9 (46 ± 18 nmol/g). These mice were fed a commercial chow diet containing α-tocopherol (30 ± 6 mg/kg diet), γ-tocopherol (10 ± 1), α-tocotrienol (3.1 ± 0.7) and γ-tocotrienol (7.4 ± 1.7). Of the vitamin E forms, brain contained only α-tocopherol (5.4 ± 0.1 nmol/g; 99.8%) and no detectable tocotrienols. In other tissues, the α-tocopherol content was higher (20 nmol/g), while each of the other forms represented about 1 % of the total (γ-tocopherol 0.2 to 0.4 nmol/g, α-tocotrienol 0.1, γ-tocotrienol 0.2). Remarkably, skin contained nearly 15% tocotrienols and 1% γ-tocopherol. The unique distribution of tocotrienols in skin suggested that they might have superior protection against environment stressors. Therefore, the penetration of topically applied vitamin E (tocotrienol enriched fraction of palm oil, TRF) and vitamin E homologue concentrations before and after exposure of skin to UV-light was assessed. 20 μL of 5% TRF in polyethylene glycol-400 (PEG) was applied to 2 skin sites and 20 μL PEG to 2 other sites. After 2 h, the skin was washed and half of the sites exposed to UV-irradiation using a solar simulator (2.8 mW/cm^2 for 29 min). The vitamin E content of hairless mouse skin was: α-tocopherol 9.0 ± 1.0 nmol/g skin, γ-tocopherol 0.44 ± 0.03, α-tocotrienol 0.48 ± 0.07, γ-tocotrienol 0.92 ± 0.03. Topical TRF enriched skin vitamin E: α-tocopherol 201 ± 70 nmol/g skin, γ-tocopherol 37 ± 15, α-tocotrienol 53 ± 25, and γ-tocotrienol 50 ± 24. After UV-irradiation, concentrations of all vitamin E homologues from both treatment areas decreased significantly ($p<0.01$), but the TRF-treated skin contained vitamin E at concentrations 7- to 30-fold higher than control values. These findings provide provocative clues on the uptake and regulation of tissue lipophilic antioxidants. The unique distribution of these antioxidant substances suggests their distribution may be dependent upon tissue-specific selective mechanisms.

Introduction

The major lipophilic antioxidant in plasma, membranes and tissues is vitamin E[1]. Vitamin E is the collective name for the 8 naturally occurring molecules (4 tocopherols and 4 tocotrienols), which exhibit vitamin E activity. Tocotrienols differ from tocopherols in that they have an isoprenoid instead of a phytyl side chain; the 4 forms of tocopherols and tocotrienols differ in the number of methyl groups on the chromanol nucleus (α- has 3, β and γ have 2, while δ has 1). Coenzyme Q (Q; reduced form: ubiquinol, oxidised form: ubiquinone) also may play an antioxidant role in membranes[2,3]. Indeed, ubiquinol protects against lipid peroxidation more efficiently than α-tocopherol in low density lipoproteins[4-6].

A very sensitive method of detection is required for the quantitation of each of these lipophilic antioxidants to determine their roles in protecting tissues against oxidative damage. We developed such a method for the simultaneous determination of individual tocopherols, tocotrienols, ubiquinols and ubiquinones[7] and measured these lipophilic antioxidants in several mouse tissues, including liver, kidney, heart, brain and skin[7].

Skin, the outermost barrier of the body, is exposed to oxidative stress from a variety of environmental insults. This oxidative damage is likely an important factor in the pathogenesis of skin cancer and photoaging[8-12]. Among the protective agents in skin are the potent lipid soluble antioxidants, vitamin E and ubiquinol[9,13-15]. During oxidative stress caused by prolonged UV-exposure, skin antioxidants are severely diminished resulting in insufficient protection and cell damage[15-17].

Topical application of vitamin E may provide an efficient way of enriching the skin with different forms of vitamin E that have a potentially higher antioxidative activity than α-tocopherol. Therefore, we measured the skin penetration of a mixture of tocopherols and tocotrienols from a tocotrienol-rich palm oil fraction (TRF), and evaluated the protection conferred by these various forms of vitamin E against UV-light induced oxidative stress[18].

This paper reviews our recent findings concerning the tissue distribution of vitamin E homologues in hairless mouse tissues. We also present our findings on the protective effects of topically applied TRF to hairless mouse skin.

Methods and materials

Materials

Highest purity solvents and reagents were used. Tocopherol standards (Covitol) were from Henkel Corporation (LaGrange, IL). TRF was kindly provided by Palm Oil Research Institute of

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Malaysia (PORIM Kuala Lumpur, Malaysia). Tocotrienols for use as standards were purified from TRF by Dr Asaf A Qureshi, University of Wisconsin (Madison, WI). Ubiquinone-9 and 10 standards were a gift from Nisshin Flour Milling Co, Ltd (Tokyo, Japan).

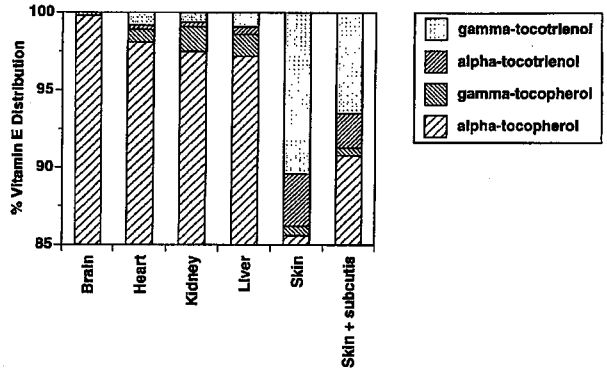
Animals

Handling and housing. Experimental procedures for animal handling were approved by the Animal Care and Use Committee of the University of California, Berkeley. Female hairless mice (SKH1, 8-12 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA) and were kept under standard light and temperature conditions. Food (Harlan Teklad Rodent Diet 8656, WI, USA) and water were provided ad libitum. Tissues were obtained from three mice, which were anaesthetised and killed by cervical dislocation.

TRF application to skin. Mice were anaesthetised by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and remained anaesthetised during the entire experimental period. Four polypropylene plastic rings (1 cm^2) were glued onto the animals' backs, then 20 µL of a 5% w/v mixture containing TRF in polyethylene glycol-400 (PEG; Sigma, St. Louis, MO). TRF was applied to the skin circumscribed by 2 rings or PEG alone to the other 2 rings. After 2 hours, the skin was washed as described by Dupuis *et al*[19], the position of the application site was marked, the plastic rings removed, and the mice exposed to UV-irradiation. Skin studies were carried out in 19 mice[18].

UV-irradiation. The mice were placed under an Oriel 1000W solar simulator (Oriel, Stratford, CT) with an output of 2.8 mW/cm^2 of UVA and UVB light (290-400 nm) and irradiated for 29 minutes (corresponding to 3 MED) on either the upper or lower back, while the other part was shielded from UV-light by covering the unexposed regions of the skin with paper and aluminium foil. After exposure, the mice were killed by neck dislocation, and the skin with subcutaneous fat were excised from the 4 exposure sites and the samples immediately frozen in liquid nitrogen. All tissues were extracted as described by Burton *et al*[20], with the exception of skin and diet samples, which were handled as described[7].

Figure 1. Percent distribution of α-tocopherol, γ-tocopherol, α-tocotrienol, and γ-tocotrienol in various hairless mouse tissues.



The fraction of the total vitamin E represented by each of the various vitamin E homologues is shown. Mice were fed chow diets.

HPLC analysis

The details of the method are reported elsewhere[7]. The HPLC system consisted of a Hewlett Packard 1050 series gradient pump, a SCL-1OA Shimadzu system controller with a SILIOA autoinjector with sample cooler, a Beckman Ultrasphere ODS C-18 column, a Hewlett Packard 1050 diode array detector and an Bioanalytical Systems LC-4B amperometric electrochemical detector with a glassy carbon electrode (0.5 V potential, full recorder scale at 50 nA). The detectors were setup in line, the eluent passing first through the diode array detector (275 nm). The mobile phase consisted of a mixture of A (80/20 v/v methanol/water and 0.2 percent w/v lithium perchlorate) and B (ethanol, reagent grade with 0.2 percent w/v lithium perchlorate) at a flow rate of 1 mL/min. The initial composition was 61% B and 39% A, after 16 minutes the mobile phase was changed linearly over a 2 min. to 100% B, which was continued for 10 min., then was changed linearly over a 2 min. to the initial composition; total run time was 40 minutes.

Quantitation was carried out by comparison of peak areas to the area of standard curves obtained with authentic compounds. For vitamin E, α- and γ-tocopherols were used as standards because the chromanol nucleus is the same in α-tocopherol and α-tocotrienol, and in γ-tocopherol and γ-tocotrienol, respectively.

Statistical analyses

Evaluation of statistical significance was carried out using SuperAnova for the Macintosh (Berkeley, CA). A p-value <0.05 was considered statistically significant.

Results

Tissue antioxidants

Vitamin E homologue concentrations were measured in various mouse tissues and in diet. In all tissues, α-tocopherol was the major form of vitamin E. As shown in Figure 1, the brain contains 99.8% α-tocopherol, while skin contains nearly 15% tocotrienols and 1% γ-tocopherol. The associated subdermal fat was not the source of these vitamin E forms because in skin samples which had the fat removed, the tocotrienol concentrations were actually higher (compare skin with skin and subcutis). In other tissues (heart, kidney, liver), each of these forms represents about 1% of the total.

The diet contained α-tocopherol (29.7 ± 6.2 mg/kg diet), γ-tocopherol (10.3 ± 1.1), α-tocotrienol (3.1 ± 0.7) and γ-tocotrienol (7.4 ± 1.7). It is likely that the tocotrienols found in mouse skin arise from the relatively low concentrations present in the diet.

Ubiquinol concentrations were highest in kidney (81 ± 29 nmol/g) and in liver (42 ± 16 nmol/g). The liver was unique in that ubiquinol-9 (42 ± 16) and ubiquinone-9 (46 ± 18) concentrations were nearly identical. Skin and brain had similar concentrations of both ubiquinols (2.2 ± 0.3 and 1.6 ± 0.1, respectively) and ubiquinone 9 (7.6 ± 1.9 and 10.2 ± 0.5, respectively); with ubiquinols representing around 20% of the total coenzyme Q. The highest ubiquinone-9 concentrations were found in heart (245 ± 22) and kidney (302 ± 124), which contain 5 to 10-fold higher amounts than did the other tissues. These ubiquinone concentrations were far greater than their respective ubiquinol concentrations.

Cutaneous absorption of tocopherols and tocotrienols following topical application

TRF treatment resulted in significant increases in vitamin E concentrations; fractional increases were greater in those forms which were present at low initial concentrations. Thus, TRF-treatment resulted in a 28 ± 16-fold increase in α-tocopherol, a 80 ± 50-fold increase in α-tocotrienol, a 130 ± 108-fold increase in γ-tocopherol and a 51± 36-fold increase in γ-tocotrienol in the skin.

Effect of UV-irradiation

To evaluate the protection by vitamin E against oxidative damage caused by UV-irradiation, the concentrations of the various vitamin E forms present in the tissue before and after UV-irradiation were measured (Figure 2). After UV-irradiation, the concentrations of all forms of vitamin E in the PEG treated-skin decreased significantly (all least square mean comparisons were $p<0.01$, except $p<0.04$ for γ-tocopherol; Figure 2, left). They also decreased significantly ($p<0.001$) after UV-irradiation as compared with non-irradiated in the TRF-treated skin (Figure 2, right). Notably, after UV-irradiation, the vitamin E homologue

concentrations were significantly higher in the TRF-treated, irradiated skin than in the PEG-treated, non-irradiated skin ($p<0.01$).

To evaluate whether the decrease in vitamin E forms in the TRF-treated samples was due to direct photodestruction of vitamin E, the effect of UV-irradiation of the TRF solution was determined *in vitro*. The percent remaining after UV-irradiation were: 86% for α-tocopherol, 83% for γ-tocopherol, 83% for α-tocotrienol, and 84% for γ-tocotrienol. These data suggest that direct photodestruction was similar for all of these vitamin E forms.

Protection of endogenous antioxidants.

To evaluate the protective effects of TRF on antioxidants that were not applied topically, ubiquinol and ubiquinone were quantitated. Ubiquinol concentrations were significantly ($p<0.002$) lower in the TRF-treated skin (1.2 ± 0.7) compared with PEG-treated skin (0.8 ± 0.6), but these differences may not be physiologically relevant because the concentrations were low with large variances. Following UV-irradiation, ubiquinol concentrations in both PEG-treated and TRF-treated skin decreased 5-fold ($p<0.001$). Ubiquinone-9 and total Q (ubiquinone-9 + ubiquinol-9) were similar in the PEG-treated and TRF-treated skin, respectively. After UV-irradiation, both ubiquinone and total Q decreased significantly ($p<0.001$) in PEG-treated and TRF-treated skin. Thus, UV-irradiation resulted in statistically significant decreases in ubiquinol, ubiquinone and total Q, which were not prevented by TRF topical application.

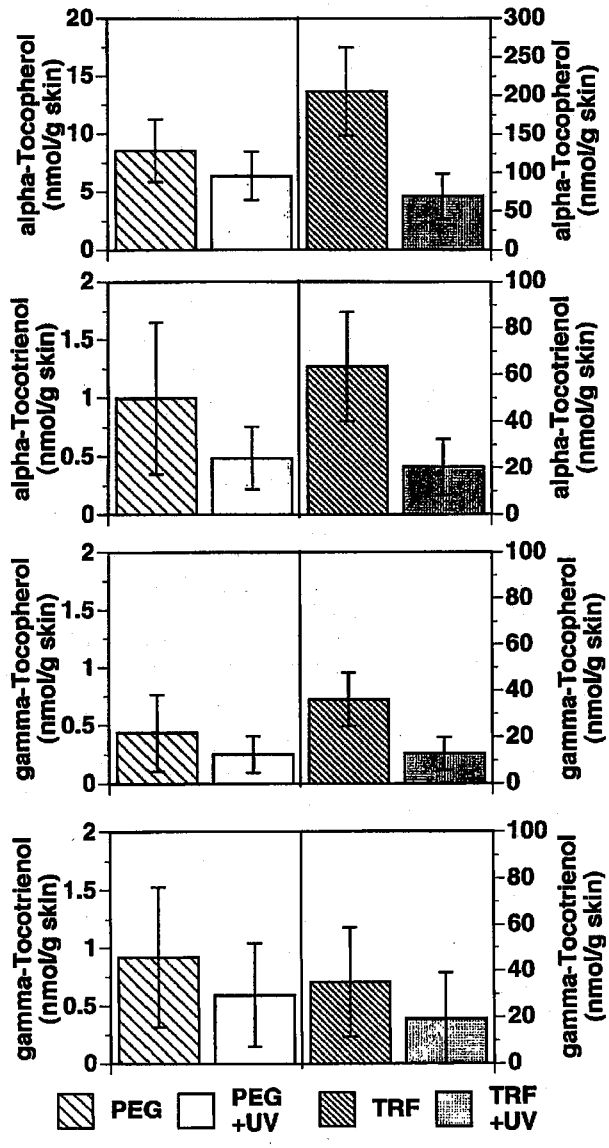
Discussion

A unique distribution of tocopherols and tocotrienols, and of coenzyme Q was found in mouse tissues. These suggest that tissues have specific mechanisms for accumulating different amounts of these antioxidants. It should be noted that the tissues analysed were obtained from mice that had been fed standard mouse chow, and were not fed diets specifically enriched in unique vitamin E forms. Hayes *et al*[21] demonstrated that tocotrienols represent only a small proportion of vitamin E in tissues from hamsters fed tocotrienol-enriched diets. The relatively high proportions of α- and γ-tocotrienols in untreated mouse skin was unexpected because 1) the mouse diets were not specially enriched with tocotrienols, and 2) the liver discriminates against tocotrienols in favour of α-tocopherol during repackaging of dietary fats into very low density lipoproteins for secretion into the circulation[1,22]. Suarna *et al*[23] demonstrated that after feeding TRF to rats, all lipoprotein classes contained tocotrienols. Apparently, transfer of tocotrienols to mouse skin must take place following absorption and transport of dietary vitamin E in chylomicrons during post-prandial chylomicron clearance and delivery of tocotrienol-containing lipoproteins[1,21,22].

Brain vitamin E is especially noteworthy because brain was found to contain only α-tocopherol. The vitamin E content of brain is spared in response to a vitamin E deficient diet[24-29] and does not markedly increase in response to vitamin E supplementation[30]. Furthermore, feeding a diet deficient in α-tocopherol, but supplemented with γ-tocopherol, did not markedly enrich the brain with γ-tocopherol[31]. Taken together these data suggest that there may be specific transport mechanisms for α-tocopherol through the blood/brain barrier.

Not only do the various vitamin E homologues have different antioxidant capabilities, they may have important physiological roles beyond their antioxidant activities[32]. For example, skin has been suggested to be an important storage site for vitamin E and a major excretory organ for this vitamin[33]. However, skin vitamin E could also have a regulatory role in maintaining barrier function. Skin contains a high proportion of tocotrienols, which could inhibit cholesterol synthesis[34]. This is important because cholesterol is a key component of the lipid barrier of the stratum corneum[35]. In addition, vitamin E may enhance penetration and resorption of skin lipids[36]. Taken together these data suggest complex regulatory mechanisms for maintenance of skin vitamin E content and composition.

Figure 2. α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol contents of murine skin.



The left panels show the vitamin E concentrations (mean ± SD, n= 19) in skin from non-irradiated or UV-irradiated hairless mice following topical application of PEG (vehicle alone). The right panels show the vitamin E concentrations in skin of hairless mice before and after UV-irradiation following topical application of TRF (in PEG). Note the different scales on the right and left y-axes. Significant decreases in each vitamin E homologue were observed after UV-irradiation both in PEG-treated and in TRF-treated skin. By least square means comparisons: PEG verses PEG+UV for α-tocopherol, $p<0.005$; α-tocotrienol, $p<0.0001$; for γ-tocopherol, $p<0.0001$; and γ-tocotrienol, $p<0.0001$. TRF verses TRF+UV for α-tocopherol, $p<0.0001$; α-tocotrienol, $p<0.0001$; for γ-toocpherol, $p<0.0001$; and γ-tocotrienol, $p<0.0001$.

After topical application of TRF, all the vitamin E forms readily penetrated into the hairless mouse skin and were present in concentrations far exceeding the baseline levels. Norkus *et al*[37] have also demonstrated that application of α-tocopheryl acetate onto hairless mouse skin results in penetration of high concentrations into skin.

Tocopherols and tocotrienols in murine skin, applied topically or derived from the diet, were significantly depleted by UV-

response to an oxidative stress, suggests that these vitamin E forms protect similarly against UV-irradiation induced damage.

A larger percentage of the various vitamin E forms remained after UV-irradiation of the PEG-treated compared with the TRF-treated skin (Figure 2). This implies a greater destruction of the various vitamin E forms in the TRF-treated skin. Whether this is due to increased free radical scavenging remains to be clarified. Localisation of TRF nearer to the upper epidermal layers in the TRF-treated skin could allow increased destruction during UV-irradiation. Alternatively, the TRF vitamin E may have penetrated the lipid components surrounding cells and thus may not be accessible to aqueous antioxidants which could recycle the vitamin E. Thus, the applied TRF may have a different behaviour during UV-exposure than the vitamin E naturally present. It should be emphasised that the skin was washed with ethanol and dried before exposure to UV-light; therefore, the vitamin E forms we have measured are not on the skin surface, but have penetrated into the skin.

Ubiquinol is the first line of defence in response to an oxidative stress[5,38]. It may readily react with the tocopheroxyl radical and be oxidised, or it may react directly with peroxyl radicals[39]. Tissues involved in detoxification, both the liver and the kidney, have extraordinarily high concentrations of ubiquinol, perhaps to protect them from radicals escaping from p450. In addition, these tissues have high concentrations of mitochondria, which could also account for their high coenzyme Q contents[2].

Coenzyme Q (ubiquinone/ubiquinol) was chosen as a marker for oxidative damage because ubiquinol is the most labile, lipid soluble antioxidant[5,38,40] and is not present in TRF. Ubiquinol is oxidised prior to α-tocopherol during UV-irradiation of skin and is substantially depleted before α-tocopherol concentrations are affected[13]. The levels of ubiquinol detected in murine skin are low; nonetheless, following UV-irradiation ubiquinol, ubiquinone and total Q all significantly decreased. Regardless of TRF application, UV-irradiation caused a loss in the total Q pool, thus depleting the skin of a vital component.

In summary, the data presented here give provocative clues to the uptake and regulation of tissue lipophilic antioxidants. This paper demonstrates not only that a variety of antioxidants are present in skin, but that topical application provides an efficient means of enriching the tissue in protective antioxidants, such as vitamin E. Furthermore, these vitamin E homologues are consumed during UV-light induced oxidative stress.

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