Ferulic Acid Stabilizes a Solution of Vitamins C and E and Doubles its Photoprotection of Skin

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Ferulic acid is a potent ubiquitous plant antioxidant. Its incorporation into a topical solution of 15% L-ascorbic acid and 1% α-tocopherol improved chemical stability of the vitamins (C + E) and doubled photoprotection to solar-simulated irradiation of skin from 4-fold to approximately 8-fold as measured by both erythema and sunburn cell formation. Inhibition of apoptosis was associated with reduced induction of caspase-3 and caspase-7. This antioxidant formulation efficiently reduced thymine dimer formation. This combination of pure natural low molecular weight antioxidants provides meaningful synergistic protection against oxidative stress in skin and should be useful for protection against photoaging and skin cancer.

Key words: antioxidant/ferulic acid/photoprotection/vitamin C/vitamin E
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Ultraviolet (UV) radiation generates oxidative stress in skin creating photodamage. Mechanistically, a photon of radiation interacts with trans-urocanic acid in skin generating singlet oxygen (Hanson and Simon, 1998). This reaction occurs maximally at about 345 nm. Singlet oxygen can generate the entire oxygen free radical cascade with oxidation of nucleic acids, proteins, and lipids, resulting in skin cancer and photoaging changes. The body deals with oxidative stress by employing a series of low molecular weight antioxidants that neutralize the reactive oxygen species before they can produce oxidative changes in tissues (Podda and Grundmann-Kollmann, 2001). In skin the predominant antioxidant is vitamin C; vitamin C protects the fluids of the body (Shindo et al, 1994). The lipid phase including cell membranes and stratum corneum is protected by vitamin E (Thiele, 2001). Plants make vitamins E and C to protect themselves from sunlight (Smirnoff et al, 2001; Munne-Bosch and Alegre, 2002). Most animals make vitamin C but humans have lost this ability; a necessary gene is mutated (Nishikimi et al, 1994). Therefore, humans typically get vitamins C and E from diet and/or vitamin supplements. Body controls related to absorption, metabolism, and distribution, however, limit the amounts that can eventually be delivered into skin (Herrera and Barbas, 2001; Padayatty et al, 2003). Moreover, when these vitamins neutralize oxidative stress in skin, they are used up (Darr et al, 1992). With daily oral supplements of 3 g vitamin C and 2 g vitamin E, protection against photodamage in skin is increased approximately 1.5 times; either vitamin alone is ineffective (Fuchs and Kern, 1998).

New formulation methods make it possible to augment protection in skin against photodamage using topical vitamins C and E, achieving significantly greater protection than ever was possible by ingestion. We have previously reported that a stable aqueous solution of 15% vitamin C (L-ascorbic acid) and 1% vitamin E (α-tocopherol) when applied topically to skin can provide 4-fold photoprotection for skin (Lin et al, 2003). The solution must be formulated at a pH of 3.5 or lower for the vitamin C to be absorbed into skin: at this pH the vitamin C is protonated and the molecule is uncharged (Pinnell et al, 2001). The formulation concentrations were maximized for percutaneous absorption. Fifteen percent L-ascorbic acid saturates skin in 3 d; its tissue half life is about 4 d (Pinnell et al, 2001). Once inside skin it cannot be removed by washing or rubbing. Vitamins C and E interact synergistically to protect each other and increase overall effectiveness (Pinnell et al, 2001).

In an attempt to improve the stability of this solution of vitamins C and E, we explored the effectiveness of a series of known low molecular weight antioxidants that are available in chemically pure form. Chemical stability was determined after 1 mo at 45°C. We have learned that addition of ferulic acid, a ubiquitous plant antioxidant, provided stability of more than 90% for L-ascorbic acid and 100% for α-tocopherol (Zielinski and Pinnell, 2004). A concentration of 0.5% gave the best combination of formulation stability and effectiveness.

We were surprised to find that in addition to improving stability, adding 0.5% ferulic acid to the solution of 15% L-ascorbic acid and 1% α-tocopherol doubled photoprotection when applied topically to skin from 4- to 8-fold. In this article, we detail these photoprotection experiments, show reduction in thymine dimer formation generated by UV radiation and demonstrate reduction of apoptosis in keratinocytes with lowered caspase-3 and caspase-7 generation.

Results

Antioxidant protection factor is designed to reflect relative photoprotection to solar-simulated radiation provided by daily topical applications of the solution for 4 consecutive days. Both 0.5% ferulic acid alone and 15% L-ascorbic acid + 1% α-tocopherol together provided about 4-fold protection (Fig 1a, b). Similar photoprotection has been previously reported for 15% L-ascorbic acid + 1% α-tocopherol (Lin et al, 2003). The combination of 15% L-ascorbic acid, 1% α-tocopherol, 0.5% ferulic acid provided approximately 8-fold protection and was statistically different than ferulic acid alone or the combination of vitamins C and E (Fig 1a, b). These observations were confirmed by colorimetric measurements of erythema (Fig 1c) and sunburn cell counts (Fig 1d). Colorimetric measurements of combination of vitamins C, E, and ferulic acid were statistically different from control and vehicle at all minimal erythema dose (MED) tested; different from ferulic acid alone at $2 \times$, $6 \times$, and $8 \times$ MED;

and different from combination vitamins C + E at $4 \times$ and $6 \times$ MED. Sunburn cell counts of combination of vitamins C, E, and ferulic acid were statistically different from control and vehicle at all MED tested and different from ferulic acid alone as well as from the combination of vitamins C + E at $2 \times$, $4 \times$, $6 \times$, and $8 \times$ MED.

In an effort to explore the mechanism of apoptosis in these experiments, western blots were carried out to determine levels of caspase-3 and its downstream effector, caspase-7. Figure 2a reveals protection by antioxidant solutions of activation of caspase-3 by $4 \times$ and $8 \times$ MED of solar-simulated radiation. Figure 2b shows relative quantification of antioxidant protection of solar-simulated radiation. With $8 \times$ MED of irradiation, protection of vitamin C, E, and ferulic acid is almost complete and is better than vitamin C and E or ferulic acid alone. Figure 2c shows activation of caspase-7 by $4 \times$ and $8 \times$ MED of solar-simulated radiation and protection by antioxidant solutions. Figure 2d shows relative antioxidant protection, which is
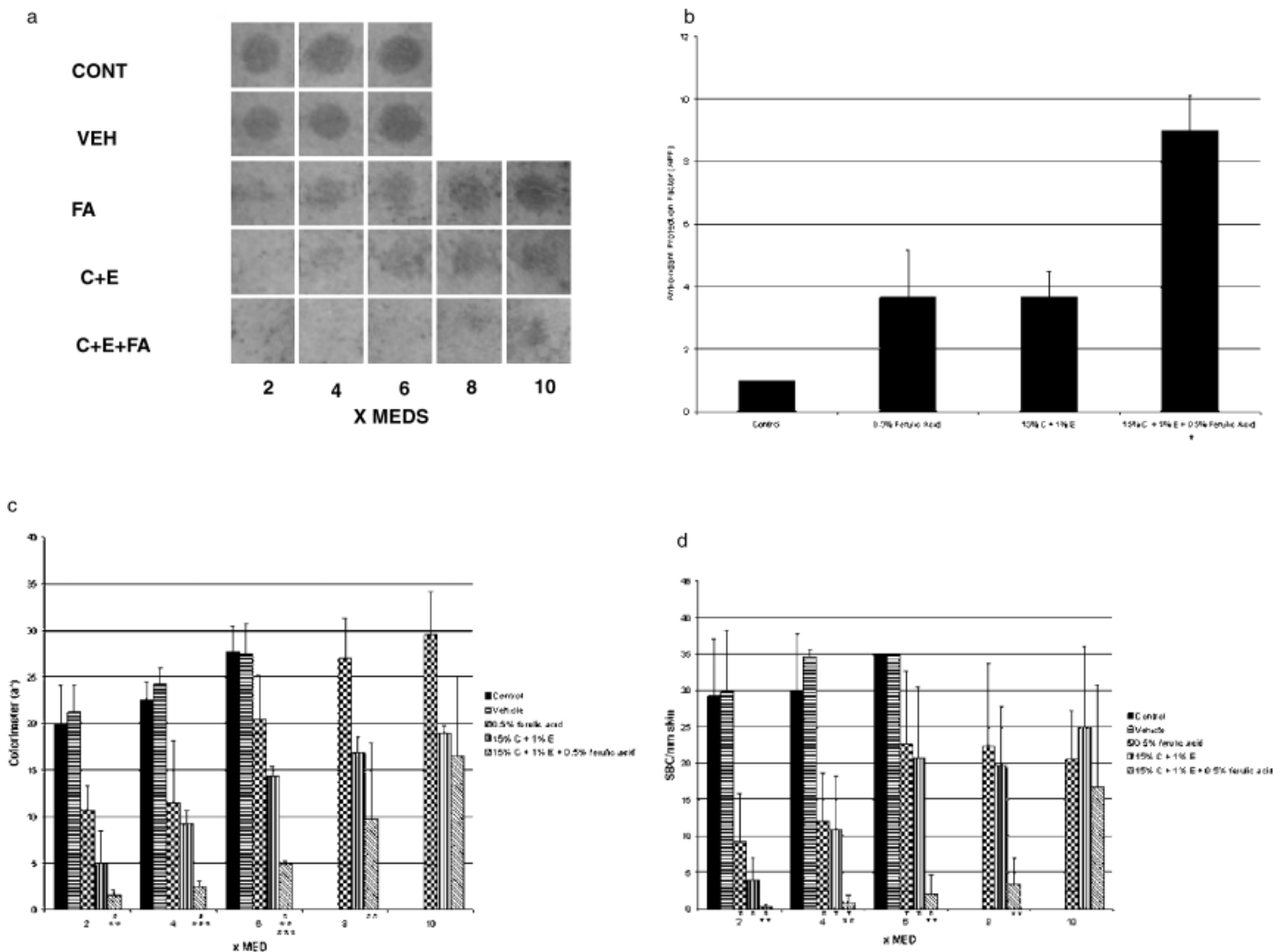

Figure 1
Photoprotection by topical antioxidant formulations. Skin was pretreated with vehicle, 0.5% ferulic acid, 15% vitamin C and 1% vitamin E, 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid and irradiated with solar-simulated radiation $2 \times$ to $10 \times$ minimal erythema dose (MED) at $2 \times$ MED intervals. Evaluation was carried out 1 d later. (a) Visual erythema of photoprotection provided by antioxidant solutions. (b) Antioxidant protection factor of antioxidant formulations. *p<0.01 vs 0.5% ferulic acid and 15% C+1% E (n=6). (c) Colorimeter measurements of photoprotection by antioxidant solutions. *p<0.05 vs control and vehicle, **p<0.05 vs 0.5% ferulic acid, ***p<0.05 vs 15% C+1% E (n=6). (d) Sunburn cell measurements of photoprotection by antioxidant solutions. *p<0.01 vs control and vehicle, **p<0.01 vs 0.5% ferulic acid and 15% C+1% E (n=6).
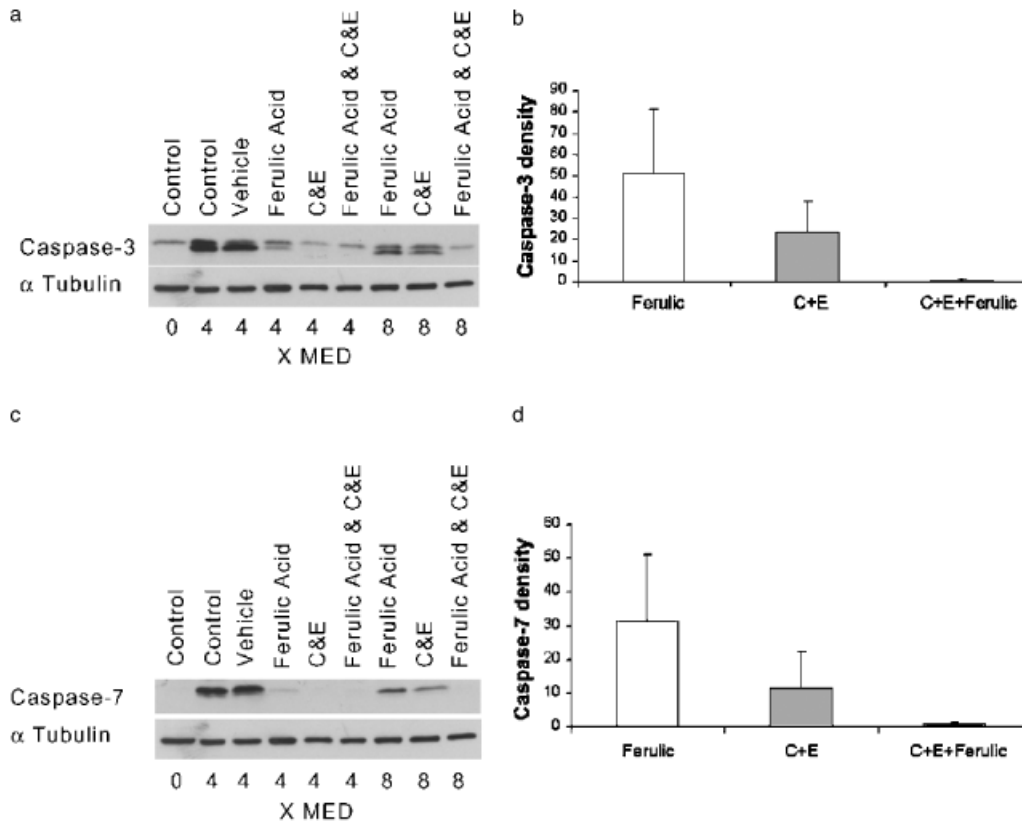
Figure 2
Activation of caspase-3 and caspase-7 by solar-simulated irradiation and relative protection by antioxidant formulations. Skin was pretreated with vehicle, 0.5% ferulic acid, 15% vitamin C and 1% vitamin E, 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid and exposed to 4 × and 8 × minimal erythema dose (MED). Levels of of caspase-3, caspase-7, and α-tubulin were determined after 24 h. Relative caspase-3 or caspase-7 levels corrected for α-tubulin are presented (n = 3). (*a*) Western blot of caspase-3 after 4 × and 8 × MED and relative antioxidant protection. (*b*) Densitometric levels of caspase-3 and relative antioxidant protection (n = 3). (*c*) Western blot of caspase-7 after 4 × and 8 × MED and relative antioxidant protection. (*d*) Densitometric levels of caspase-7 and relative antioxidant protection (n = 3).

virtually complete by vitamins C, E and ferulic acid. Figure 3 shows immunohistochemistry of activation of caspase-3 by 4 × MED of solar-simulated light. Activation occurs in both epidermis and dermis (Fig 3*a–c*); in epidermis, activation is particularly strong in the basal layer. Ferulic acid alone (3b) and vitamins C + E (3c) provide partial protection but vitamins C, E, and ferulic acid (3d) provides virtually complete protection.

We have previously demonstrated that topical vitamins C + E could prevent UV-induced thymine dimer formation (Lin *et al*, 2003). In order to determine whether the addition of ferulic acid augmented this protection, we investigated the relative dose–response protection of these formulations (Fig 4). With 4 × MED of solar-simulated irradiation, both control and vehicle-treated skin showed virtually uniform nuclear fluorescence in both epidermis and papillary dermis (Fig 4*a*). After 4 × MED, ferulic acid-treated skin was still positive in approximately 25% of both epidermis and dermis (data not shown). As previously reported, vitamins C + E almost completely protected skin irradiated with 4 × MED (data not shown). After 8 × MED, ferulic acid-treated skin was about one third positive (Fig 4*b*) and vitamin C + E-treated skin was about 15% positive and fluorescence was less intense (Fig 4*c*). Skin treated with vitamins C, E, and ferulic acid was completely negative (Fig 4*d*).

Discussion

Ferulic acid not only provides increased stability to a solution of vitamins C + E, but also adds a substantial synergistic photoprotection, essentially doubling its efficacy. Moreover it provides additional protection against thymine dimer formation that should prove useful for prevention of skin cancer. These studies support the hypotheses that UV radiation produces apoptosis by triggering the caspase cascade in both epidermis and dermis, and topical vitamins C, E, and ferulic acid can protect against caspase activation.

Ferulic acid is a potent phenolic antioxidant found ubiquitously and at high concentrations in plants (Graf, 1992; Rice-Evans *et al*, 1996; Ou and Kwok, 2004). It serves to cross-link polysaccharides and proteins during lignin cell wall synthesis (Wallace and Fry, 1994; Mathew and Abraham, 2004) and may be important for the health effects of bran; corn bran contains 3.1% ferulic acid (Mathew and Abraham, 2004). It is abundant in the diet and has low toxicity (Ou and Kwok, 2004; Zhao *et al*, 2004). Ferulic acid is a potent antioxidant with synergistic interactions with ascorbic acid (Trombino *et al*, 2004). It readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential (Graf, 1992). Ferulic acid protected membranes from lipid peroxidation and neutralized alkoxyl and peroxyl radicals (Trombino *et al*, 2004). It protected against iron-induced oxidative damage (Hynes and O'Coinceanainn, 2004). Ferulic acid scavenged hydroxyl radical (Ogiwara *et al*, 2002; Wenk *et al*, 2004), nitric oxide (Wenk *et al*, 2004), peroxynitrite (Pannala *et al*, 1998; Dinis *et al*, 2002), and superoxide radical (Kaul and Khanduja, 1999; Kikuzaki *et al*, 2002). It was antimutagenic (Yamada and Tomita, 1996; Ferguson *et al*, 2003), protected against menadione-induced oxidative DNA damage (Burdette *et al*, 2002) and demonstrated anticarcinogenic effects in animal
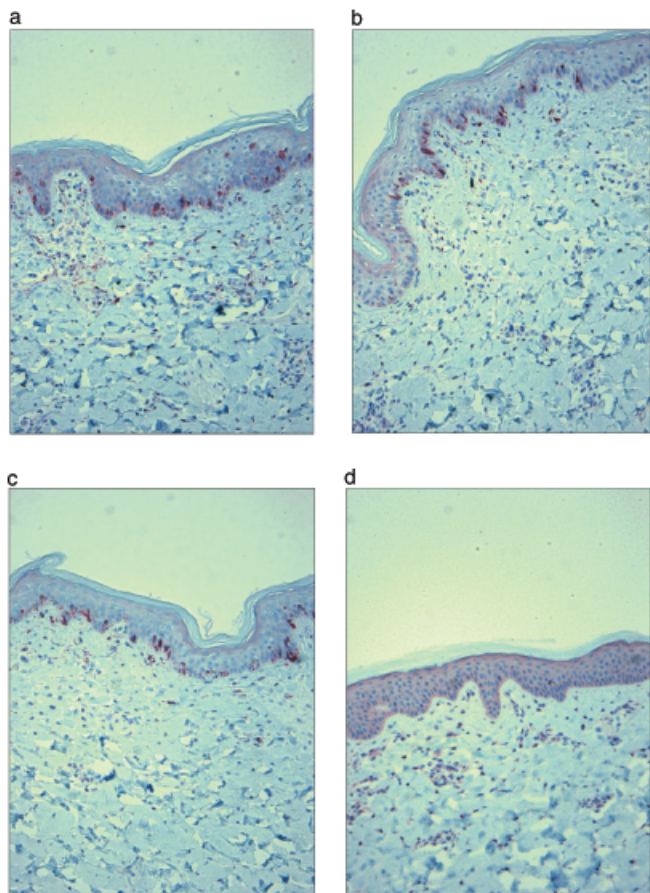
Figure 3
Immunochemistry of activated caspase-3 after solar-simulated irradiation. Skin was pretreated with 0.5% ferulic acid, 15% vitamin C and 1% vitamin E, or 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid and exposed to 4 × minimal erythema dose. After 24 h formalin-fixed tissues were stained with antibodies to activated caspase-3. (*a*) Vehicle-treated irradiated skin; (*b*) 0.5% ferulic acid-treated irradiated skin. (*c*): 15% vitamin C and 1% vitamin E-treated irradiated skin; (*d*) 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid-treated irradiated skin.

Figure 4
Immunochemistry of thymine dimers after solar-simulated irradiation. Skin was pretreated with vehicle, 0.5% ferulic acid, 15% vitamin C and 1% vitamin E, or 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid and exposed to 4 × or 8 × minimal erythema dose (MED). After 24 h formalin-fixed tissues were stained with antibodies to thymine dimers. (*a*) Vehicle-treated skin after 4 × MED; (*b*) 0.5% ferulic acid-treated skin after 8 × MED; (*c*) 15% C and 1% vitamin E-treated skin after 8 × MED; (*d*) 15% vitamin C and 1% vitamin E, and 0.5% ferulic acid-treated skin after 8 × MED.

models of pulmonary (Lesca, 1983) and colon carcinoma (Kawabata *et al*, 2000; Wargovich *et al*, 2000). Topical application inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase activity and decreased TPA-induced skin tumor formation (Huang *et al*, 1988). Topical application of ferulic acid inhibited UVB-induced erythema (Saija *et al*, 2000). In a mouse model of multiple sclerosis, oral ferulic acid had a striking effect on syncytin-mediated inflammation and death of oligodendrocytes induced by redox reactants (Antony *et al*, 2004). Ferulic acid absorbs UV radiation with an absorption maximum at 307 nm (log $e = 4.19$) (Graf, 1992). Theoretically this absorption could result in a sunscreen effect providing topical protection against UV radiation. Our studies revealed no evidence of a dose–response effect to support a sunscreen mechanism (Fig 5).

The mechanism of ferulic acid's stabilizing effect on vitamins C and E is unknown. It would not be expected to directly protect these vitamins since its redox potential (0.595) is appreciably higher than vitamin C (0.282) or vitamin E (0.4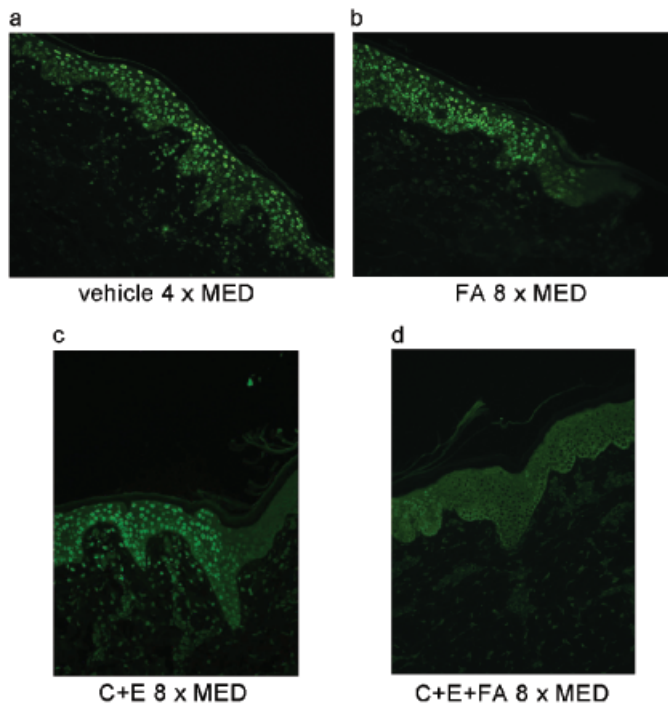8) (Lu and Liu, 2002). Since it provides protection against vitamin C degradation, it may preferentially interact with pro-oxidative intermediates, or serve as a sacrificial substrate. It is also possible that its interactions may be enhanced at the low pH of the formulation. Ferulic acid's effect on photoprotection is most likely related to its antioxidant activity. It had no appreciable effect on ascorbic acid absorption (data not shown).

Ferulic acid augments the protection of vitamins C + E previously demonstrated to prevent UV-induced thymine dimer formation when applied topically to skin (Lin *et al*, 2003). A recent study of actinic keratoses and squamous cell carcinomas of skin using laser capture microdissection reveals 8-oxo guanine mutations in the basal germinative layer and thymine dimer mutations at suprabasal locations (Agar *et al*, 2004). The results support the hypothesis that UVA-induced oxidative DNA modifications are responsible for the carcinogenic mutations in stem cells and UVB-induced mutations promote the carcinogenic process. The hypothesis fits with the superficial penetration of UVB and the deeper penetration of UVA into skin. Although we have not yet been able to measure the effect of antioxidants on UV-induced 8-oxo guanine formation, protection would be predicted. The demonstrated protection of topical application of vitamins C, E, and ferulic acid against UV-induced thymine dimer formation supports its use for the prevention of skin cancer.

Recent studies substantiating the shortcomings of sunscreen protection support the need for a different approach
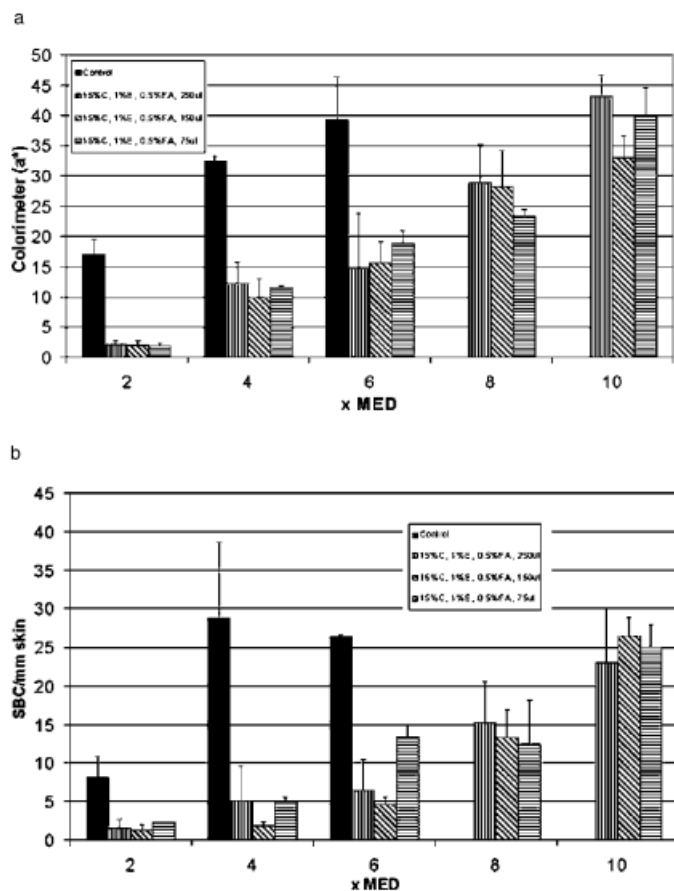
Figure 5
Effect of dosage on photoprotection provided by vitamins C, E, and ferulic acid. Skin was pretreated by 75, 150, and 250 μL of 15% vitamin C, 1% vitamin E, and 0.5% ferulic acid and irradiated with solar-simulated radiation 1 × to 5 × minimal erythema dose (MED) at 1 × MED intervals. Evaluation was carried out 1 d later. (*a*) Colorimeter measurements of photoprotection by vitamins C, E, and ferulic acid (n = 3). (*b*) Sunburn cell measurements of photoprotection by vitamins C, E, and ferulic acid (n = 3).

to photoprotection. Sun protection factor is measured at 2 mg per cm^2, yet in actual use, sunscreen application is only 0.4–0.5 mg per cm^2 (Wulf *et al*, 1997; Autier *et al*, 2001). Since SPF is an exponential measurement, at reduced levels, relative photoprotection of any sunscreen is no more than 3–4-fold. In addition, SPF is a measurement of UVB only and reveals nothing about UVA photoprotection, protection necessary to protect against oxidative stress. Indeed a recent study of three high SPF broad-screen sunscreens revealed that at 2 mg per cm^2 application, UVA-induced free radical formation was reduced only 55% with even worse protection when application levels were decreased (Haywood *et al*, 2003). Sunscreens are designed to be shields for the skin, protecting the skin by absorbing harmful UV radiation. Since they work at the surface of the skin, they are easily removed by washing or rubbing. Antioxidants, in contrast, are designed to work not only at the skin's surface but also inside skin. Following topical application, once the skin is saturated with L-ascorbic acid, it remains with a half-life of about 4 d (Pinnell *et al*, 2001). Approximately one third of UVA-induced oxidative stress in skin occurs at the surface of skin (Ou-Yang *et al*, 2004).

Vitamin E is delivered to the surface of skin through sebum to protect the stratum corneum from this oxidative insult (Thiele, 2001). Direct topical application of antioxidants would be expected to facilitate this protection. Using both a properly formulated topical combination antioxidant product containing vitamins C and E with ferulic acid, and also a broad-spectrum sunscreen, would be expected to provide optimal photoprotection. Since they work by different mechanisms, they should be supplemental (Darr *et al*, 1996).

Materials and Methods

L-ascorbic acid, DL-α-tocopherol, and *trans* ferulic acid were purchased from Sigma (St Louis, Missouri). Aqueous solutions were prepared in a vehicle containing diethylene glycol monoethyl ether, 1,2-propanediol, Brij-35, and phenoxyethanol at pH 3.

Treatment and irradiation procedure The experimental design has previously been published in detail (Lin *et al*, 2003). Experiments were performed on weanling white Yorkshire pigs in accord with the guidelines prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (National Institutes of Health, publication No. 86–23, revised 1996). Unless otherwise noted, 500 μL aliquots of vehicle; 0.5% *trans* ferulic acid; 15% L-ascorbic acid, 1% DL-α-tocopherol, and 15% L-ascorbic acid, 1% DL-α-tocopherol, 0.5% *trans* ferulic acid were applied to patches of back skin (7.5 × 10 cm) daily for 4 d. A 1000 W solar simulator (Lightning Cure 200, Hamamatsu, Japan) fitted with a WG295 Schott filter to eliminate wavelengths less than 295 nm delivered UV radiation to the skin's surface through a liquid light guide at an intensity of 5 mW per cm^2 of UVB and about 40 mW per cm^2 of UVA as measured by a radiometer (IL1700, International Light, Newburyport, Mississippi). MED was determined as the lowest dose resulting in erythema with perceptible borders (40 mJ per cm^2 of UVB). Each patch was given solar-simulated irradiation in triplicate from 2 × to 10 × MED at 2 × MED intervals. Evaluation was carried out 24 h later.

Evaluation of antioxidant protection factor, erythema, and sunburn cells Antioxidant protection factor was calculated as the ratio of the MED in antioxidant-treated skin in comparison with untreated skin. Erythema was measured by colorimeter evaluation in the "a" mode (ColorMouse Too, Color Savvy Systems Ltd, Springboro, Ohio) of 8 × 12 in enlargements of skin photographs. Each spot and adjacent unirradiated skin was measured in triplicate. The difference between irradiated and unirradiated skin determined the erythema. Sunburn cells were determined in formalin-fixed 8 mm punch biopsy sections stained with hematoxylin and eosin. When irradiation damage was extensive, the number 35 sunburn cells per mm were used as an upper limit.

Evaluation of caspase-3 and caspase-7 For western blotting, cell protein was extracted from freeze-fractured skin in a solution containing 1% NP-40%, 1% sodium deoxycholate, 0.3% SDS, 0.15 M NaCl, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium phosphate, pH 7.2, and a pre-formed protease inhibitor mixture (Sigma). Protein extract (50 μg) mixed with mercaptoethanol and SDS-PAGE sample buffer was heated at 100°C for 5 min, and separated on 12% SDS-polyacrylamide gel, electrotransferred to PVDF membrane (Millipore, Bedford, Massachusetts), blocked in TBS (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.1% Tween-20% and 5% milk for 1 h at room temperature and incubated with primary antibody: rabbit anti-human cleaved caspase-3, or cleaved caspase-7 diluted 1:1000 (Cell Signaling Technology, Beverly, Massachusetts) overnight at 4°C. After reaction with horseradish peroxidase-conjugated goat anti-rabbit IgG, immunocomplexes were visualized using ECL (Amersham Pharmacia

Biotech, Piscataway, New Jersey). For internal control, the blots were stripped and reprobed with anti-α tubulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California). Densitometry was performed using Kodak ID image analysis software (Kodak, Rochester, New York).

Formalin-fixed skin was prepared for caspase-3 or caspase-7 immunocytochemistry by following manufacturer's protocol. Briefly, sections were deparaffinized/hydrated, heated in 10 mM sodium citrate buffer (pH 6) for antigen unmasking, incubated in 1% hydrogen peroxide, followed by incubation with 5% horse serum. Sections were incubated with primary antibody solution (1:200 dilution) overnight at 4°C, followed by biotin-labeled anti-rabbit IgG secondary antibody according to ABC biotin/avidin method (Vector Laboratories, Burlingame, California). Finally, sections were incubated in peroxidase substrate solution (DAB, Vector Laboratories), and counterstained with hematotoxylin.

Evaluation of thymine dimers The procedure was modified from the method described by Mitchell *et al* (2001). Briefly, sections were deparaffined/rehydrated and washed with phosphate-buffered saline (PBS), denatured in 0.1 N NaOH/70% EtOH for 5 min, dehydrated in EtOH, air dried, and incubated with protease XXV (Labvision, Fremont, California) at 37°C for 7 min. After washing with PBS, sections were blocked with 5% goat serum for 20 min, washed, incubated with anti-thymine dimer, clone KTM53 diluted 1:200 (Kamiya Biomedical Company, Seattle, Washington) at 37°C for 1 h, followed by anti-mouse IgG conjugated with fluorescein isothiocyanate diluted 1:200 at 37°C for 30 min. Sections were visualized using Olympus BX41fluorescence microscope coupled with a Q-Fire camera.

Statistics Results are expressed as mean ± SD. The p-values were calculated by two-tailed Student's *t*-test.

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