Ozone depletes tocopherols and tocotrienols topically applied to murine skin

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Abstract To evaluate ozone damage to hairless mouse skin, two parameters of oxidative damage, vitamin E depletion and malondialdehyde (MDA) production, were measured in vitamin E-enriched and in control skin from mice exposed to ozone (10 ppm). A 5% vitamin E solution (tocotrienol-rich fraction, TRF) in polyethylene glycol (PEG) was applied to 2 sites on the back of hairless mice, PEG to 2 sites. After 2 h, the sites were washed, one of each pair of sites covered and the mice exposed ozone for 2 h. Ozone exposure (compared with covered sites) increased epidermal MDA in PEG-treated sites, while vitamin E was unchanged. In contrast, ozone exposure significantly depleted vitamin E in TRF-treated sites, while significant MDA accumulation was prevented. This is the first demonstration that ozone exposure causes damage to cutaneous lipids, an effect which can be attenuated by vitamin E application.

Key words: Hairless mice; Vitamin E; Antioxidant; Malondialdehyde; Epidermis; Cutaneous lipid

1. Introduction

Ozone is the major air pollutant in photochemical smog. Since half the US population lives in areas exceeding the US National Ambient Air Quality Standard (0.12 ppm averaged over a 1 h period) [1], the presence of ozone in the air poses significant concern [2].

Ozone exposure causes oxidation and peroxidation of biomolecules both directly and/or via secondary products of ozone reactions [3–8]. One of the most important mechanisms of ozone injury is peroxidation of lipids, especially unsaturated fatty acids [5,7,8]; in vitro, vitamin E appears to prevent the propagation of this reaction [6]. Although α-tocopherol has the highest biologic activity of the various vitamin E isomers [9], the properties of some of the other isomers suggest that they might be more protective against ozone-induced damage. For example, α-tocotrienol has a higher antioxidative activity than α-tocopherol against Fe^{2+}/ascorbate and Fe^{2+}/NADPH-induced lipid peroxidation in rat liver microsomes [10]. Alternatively, ozone might attack the chromanol nucleus of γ-tocotrienol or γ-tocopherol in an analogous manner to that described for nitrogen dioxide [11].

Skin is the organ the most directly exposed to ozone. In fact, ozone is probably the most reactive environmental pollutant to which skin is routinely exposed. Unlike the lung [4], very little attention has been paid to the potential effects of environmental oxidant pollutants on cutaneous tissues. This is somewhat surprising since skin contains peroxidizable lipids and these constituents are in part responsible for the cutaneous permeability barrier [12].

A variety of enzymatic and non-enzymatic antioxidants protect skin against oxidative stress [13–17]. Among these is vitamin E. Of the vitamin E isomers, skin α-tocopherol concentrations are much higher than γ-tocopherol or α- and γ-tocotrienols [18]. However, skin can be enriched by topical application of these vitamin E isomers [19].

This study tested the hypotheses that (1) ozone attacks skin lipids and lipophilic antioxidants and (2) topical application of a mixture of vitamin E forms, including tocopherols and tocotrienols from a tocotrienol-rich palm oil fraction (TRF), ameliorates this oxidative damage.

2. Materials and methods

2.1. Chemicals

All chemicals used were of the highest grade available. Tocopherol standards were provided by Henkel Corporation (LaGrange, IL). TRF was kindly provided by PORIM (Kuala Lumpur, Malaysia). Tocotrienols for use as standards were purified from TRF by Dr. Asaf A. Qureshi, University of Wisconsin (Madison, WI). TRF vitamin E are extracted from palm oil and so are in the 'natural' configuration – both α- and γ-tocopherols have 2R, 4′R, 8′R-stereochemistry; both α- and γ-tocotrienols have 2R stereochemistry.

2.2. Animals

Animal care, handling, and experimental procedures were carried out as described in the animal use protocol approved by the Animal Care and Use Committee of the University of California, Berkeley, CA. Hairless mice (females, between 8 and 10 weeks old, Charles River Laboratories, Wilmington, MA, USA) were housed under standard light and temperature conditions. Food (Harlan Tecklad Rodent Diet #1846, Madison, WI, USA) and water were provided ad libitum. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Nembutal, Abbott Laboratories, North Chicago, IL) and remained anesthetized during the entire experimental period.

2.3. Vitamin E application

A 5% w/v solution of TRF was prepared in polyethylene glycol-400 (PEG; Sigma, St. Louis, MO). Mice were anesthetized, then 4 polypropylene plastic rings (1 cm^2) were glued onto the animals' backs, subsequently TRF solution (20 μl) was applied to 2 rings and PEG to the other 2 rings. After 2 h, the treated areas were washed as de-

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylene diamine tetraacetic acid; HPLC-EC, high pressure liquid chromatography with electrochemical detection; PEG, polyethylene glycol-400; TRF, tocotrienol-rich palm oil fraction

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scribed by Dupuis et al. [20]. Briefly, the skin was rinsed 3 times with 300 μl ethanol:water (95:5), then twice with water alone and dried with a cotton tip. After washing, the location of the application site was marked and the plastic rings removed; half of the sites were covered with a piece of Kimwipe tissue (Kimberly Clarke, Atlanta, GA) and sealed with cellophane tape. The Kimwipe prevented injury to the skin during the removal of the adhesive tape following exposure to either air ($n=3$, control) or ozone ($n=4$) (see below).

2.4. Ozone exposure

Ozone was produced from oxygen by electric discharge (Sander ozonizer model IV, Eltze, Germany). The ozone was then mixed with filtered (ozone-free) ambient air and allowed to flow into a stainless steel exposure chamber at a constant rate (200 l/min). The concentration in the exposure chamber was adjusted to 10 ppm and continuously monitored with an ozone analyzer (Dasibi model 1003-AH, Glendale, CA). The ozone chamber provided a maximum space for 4 animals during the 2 h ozone exposure. Control mice were treated identically in terms of anesthesia and animal handling, but were kept under air (0 ppm ozone).

After air or ozone exposure, the mice were given another dose of pentobarbital, allowed to rest for 30 min, then were killed by cervical dislocation. The skin was excised, the subcutaneous fat removed with a scalpel, then punch biopsies were taken and the samples immediately frozen in liquid nitrogen. The skin samples were stored for no longer than a week at −80°C.

2.5. Vitamin E analysis

Tocopherols and tocotrienols were extracted from full thickness skin, as described [21,22]. Briefly, the skin sample was weighed (approximately 20 mg), ground under liquid nitrogen, then homogenized in a Potter-Elvehjem homogenizing tube with 2 ml buffer (10 mM phosphate, 130 mM NaCl, 1 mM EDTA, pH 7.0) and 50 ml BHT (1 mg/ml) and extracted after addition of 1 ml of 0.1 M SDS, 2 ml ethanol and 2 ml hexane. An appropriate aliquot of hexane was used for HPLC analysis, as described [23]. The electrochemical detector was operated with a 0.5 V potential, full recorder scale at 50 nA for quantitation of α- and γ-tocopherols and α- and γ-tocotrienols. Authentic compounds were used to generate standard curves.

2.6. Lipid peroxidation

Fluorimetric detection of the malondialdehyde-thiobarbituric acid adduct (MDA-TBA) was performed after HPLC separation of the TBA-reactive substances, based on methods for MDA determination in plasma and other body tissues [19–21]. After weighing, the scraped mouse epidermis was extracted with 2 ml methanol, 2 ml 15% SDS solution, 50 μl 10% BHT in ethanol and 4 ml chloroform. An aliquot of the chloroform was dried and the residue resuspended in 400 μl 15% SDS and incubated with 250 μl 0.375% TBA and 200 μl 1.22 M phosphoric acid for 30 min at 100°C, followed by addition of 380 μl methanol and 20 μl 1 N NaOH. After centrifugation, 100 μl supernatant were injected into the HPLC system, which consisted of a 114 M Solvent Delivery Module pump (Beckman, Fullerton, CA), an Alltima C18 column and a Hitachi (Hitachi Ltd. Tokyo, Japan) F-105 fluorescence spectrophotometer (excitation 532 nm and emission 553 nm). The mobile phase consisted of 60% methanol and 40% 50 mM NaH_2PO_4, pH adjusted to 5.5, at a rate of 0.9 ml/min. MDA standards (ranging from 0.5 to 10 pmol) were prepared using dilutions of 1,1,3,3-tetramethoxypropane. Samples and standards were analyzed in duplicate.

2.7. Statistical analysis

All statistical analyses were carried out using SuperAnova (Abacus Concepts, Inc., Berkeley, CA) for the Macintosh (Apple Computers, Cupertino, CA). Analyses included: one-factor ANOVA (air vs. ozone); two-factor ANOVA with 2 within groups repeated measures (PEG vs. TRF, and covered vs. exposed) with least square means comparisons. Data were log-transformed to equalize variances between TRF- and PEG-treated sites. A P-value <0.05 was considered statistically significant. Values are given as means ± S.D.

3. Results

3.1. Antioxidants in murine skin

To investigate the susceptibility of skin to ozone damage, a study design was planned using covered and uncovered skin sites, such that each animal would serve as its own control. Therefore, we first evaluated whether covering the skin changed the antioxidant composition. PEG was applied to 2 sites and TRF to 2 sites, the compounds were allowed to penetrate for 2 h, then the skin was washed. Subsequently, half of the sites were covered with tissue paper and sealed with cellophane tape for 2 h during which time the mice were exposed to air. Tocopherols and tocotrienols in PEG-treated air-exposed skin were within the range of previous reports from our laboratory [19,24], while covering the skin significantly increased vitamin E contents (Table 1). TRF treatment resulted in significant increases in the concentrations of all vitamin E forms, as reported previously [19]. However, covering the TRF-treated sites resulted in significantly lower concentrations of vitamin E than in the uncovered sites.

To evaluate whether the various vitamin E forms penetrated murine skin differently, the percentage distribution of each of the vitamin E homologues in the TRF suspension was compared to its percentage distribution in skin treated with TRF or vehicle (PEG) alone (Fig. 1). The percentage distribution of vitamin E forms that penetrated the skin (above background concentrations) was significantly different from their distribution in the TRF suspension – a higher percentage of α-tocopherol was found in TRF-treated skin than was present in TRF suspension ($P<0.01$).

3.2. Ozone exposure depletes vitamin E-enriched skin

To evaluate skin damage caused by ozone, vitamin E concentrations were used as a marker of oxidative damage. The concentrations of the various vitamin E isomers were measured in the 4 sites (PEG-treated covered and uncovered, TRF-treated covered and uncovered) in mice exposed to ozone (10 ppm for 2 h) (Fig. 2). Surprisingly ozone had no measurable effect on the inherent vitamin E concentrations measured in whole thickness skin. The vitamin E contents of sites from ozone-exposed PEG-treated covered and uncovered were similar (Fig. 2), and these values were similar to those in air-exposed PEG-treated, mice (Table 1). Thus, ozone exposure did not deplete inherent skin vitamin E.

By contrast to inherent vitamin E, skin with topically applied vitamin E was readily susceptible to ozone damage (Fig. 2). Ozone exposure significantly depleted TRF-treated skin vitamin E: α-tocopherol ($P<0.01$), γ-tocopherol ($P<0.05$), α-tocotrienol ($P<0.05$) and γ-tocotrienol ($P<0.04$). None-

Table 1
Vitamin E contents (pmol/mg skin) of murine skin following topical application of PEG or TRF, then covered or exposed to air

Applied	Treatment	α-Tocopherol	γ-Tocopherol	α-Tocotrienol	γ-Tocotrienol
PEG	Covered	29 ± 4	2.3 ± 0.3	6.4 ± 1.1	5.6 ± 2.5
PEG	Exposed	15 ± 3	0.5 ± 0.2	2.1 ± 0.8	2.5 ± 1.3
TRF	Covered	138 ± 64	17.1 ± 8.6	45.0 ± 21.7	39.5 ± 26.5
TRF	Exposed	357 ± 210	44.8 ± 28.3	113.7 ± 73.0	109.4 ± 74.7

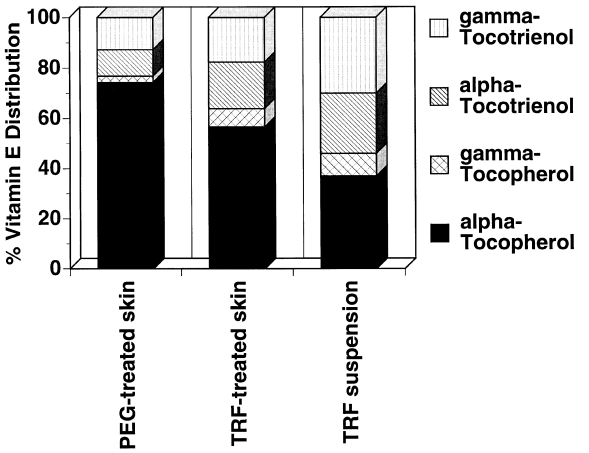

Fig. 1. Vitamin E distribution. The percent of each of the vitamin E forms found in PEG-treated skin, in TRF-treated skin and in the TRF suspension are shown. The TRF-treated skin contained a significantly higher percentage of α-tocopherol ($P < 0.01$) than did the applied TRF.

theless, after ozone exposure, the concentrations of all vitamin E forms were the same (γ-tocotrienol, $P < 0.06$), or significantly higher in the TRF-treated ozone exposed-skin than in the PEG-treated, covered-skin (α-tocopherol: $P < 0.02$, γ-tocopherol: $P < 0.04$, α-tocotrienol: $P < 0.04$).

Covering the skin during ozone exposure prevented vitamin E depletion in the TRF-treated sites. The ozone-exposed, covered TRF-treated sites (Fig. 2) contained similar vitamin E concentrations to those exposed to air (Table 1). Thus, the 2 h ozone exposure, did not result in systemic effects that altered skin vitamin E concentrations.

3.3. Oxidative damage to skin lipids

To evaluate ozone damage to skin lipids, MDA was measured in the epidermal layers of skin from the 4 sites (PEG-treated covered and uncovered, TRF-treated covered and uncovered) in mice exposed to ozone (10 ppm for 2 h). Here a dramatic increase ($P < 0.01$) in the epidermal MDA of ozone-exposed PEG-treated skin was observed (Fig. 3). The smaller increase in MDA observed in the ozone-exposed TRF-treated site was not statistically significant. These data suggest that ozone can oxidize the lipids present in the upper layers of skin and that to some degree this damage is ameliorated by applied vitamin E.

4. Discussion

Two major physiological implications arise from this study. (1) Ozone exposure (10 ppm for 2 h) causes lipid peroxidation in cutaneous tissues and (2) prior application of vitamin E can ameliorate some of the resultant oxidative damage.

Although ozone exposure similarly depleted all the various forms of vitamin E (α-tocopherol, γ-tocopherol, α-tocotrienol, and γ-tocotrienol) that were applied topically, these forms in whole thickness skin were not significantly depleted. The observation that ozone depleted applied, but not inherent, vitamin E suggests that ozone probably attacks the outermost layers of skin, where the applied forms of vitamin E are presumably the most concentrated. The supposition that ozone effects are primarily on the skin surface is further substantiated by the observation that epidermal MDA was increased by ozone exposure and was reduced in TRF-treated skin. Analyses of antioxidant contents of uppermost skin layers (e.g. epidermis, stratum corneum) may be necessary to observe ozone-induced antioxidant depletion in cutaneous tissues.

Environmental ozone toxicity may result from the effects of a cascade of products that are produced in the reactions of ozone with primary target molecules that lie close to the air/tissue boundary. These products, which have a lower reactivity and longer lifetime than ozone itself, can transmit the effects of ozone beyond the air/tissue interface [8,25]. To investigate whether the observed changes in vitamin E concentrations could, besides direct ozone effects to the skin, be due to effects of secondary products of ozone formed in the lung and brought to the dermis via blood circulation, one site of the mouse's back skin was covered by tape during ozone exposure. Since no significant changes in vitamin E concentrations were observed compared with air treatment (compare Fig. 1 with Table 1), we conclude that the observed antioxidant depletion in skin is primarily due to direct ozone effects to the skin. Furthermore, the high concentrations of plasma antioxidants [26] make it unlikely that significant amounts of secondary ozone products were transmitted from the respiratory tract to the skin via the dermal circulation during the short experimental period.

The use of covered skin allowed animals to serve as their own controls during ozone exposure. However, the covered PEG-treated skin contained *higher* vitamin E concentrations than air-exposed skin, while the covered TRF-treated skin

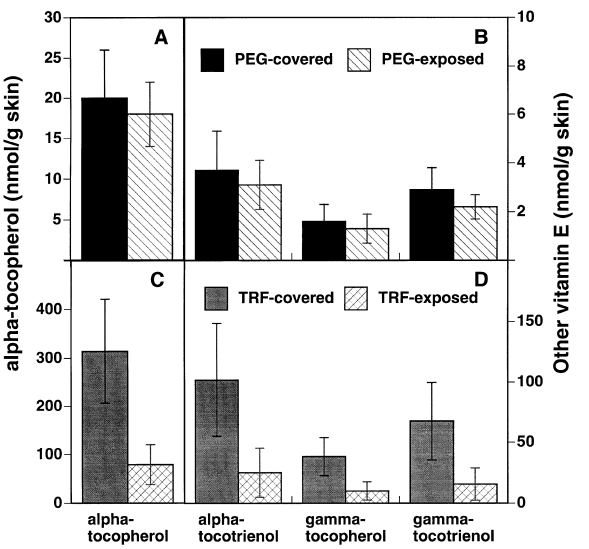

Fig. 2. Vitamin E content of murine skin after 2 h ozone exposure. The upper panels show the concentrations (mean ± S.D., n=4) of (A) α-tocopherol and (B) α-tocotrienol, γ-tocopherol, and γ-tocotrienol in skin from covered or ozone-exposed sites after topical application of PEG. The lower panels show the concentrations of (C) α-tocopherol and (D) α-tocotrienol, γ-tocopherol, and γ-tocotrienol from covered or ozone-exposed sites after topical application of TRF. Significant decreases in each of the homologue concentrations were observed after ozone exposure in TRF-treated skin. By least square means comparisons: PEG-covered vs. PEG-exposed, NS. TRF-covered vs. TRF-exposed for α-tocopherol, $P < 0.01$; for α-tocotrienol, $P < 0.05$; γ-tocopherol, $P < 0.05$; and γ-tocotrienol, $P < 0.04$. Covered sites PEG vs. TRF for α-tocopherol, $P < 0.002$; for α-tocotrienol, $P < 0.01$; γ-tocopherol, $P < 0.01$; and γ-tocotrienol, $P < 0.01$.

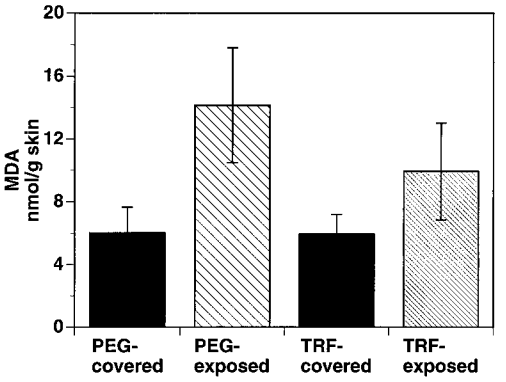

Fig. 3. MDA in murine skin. The MDA concentrations (mean ± S.D.) in mouse epidermis following application with PEG or TRF, covered or not, and exposed to ozone ($n = 4$). Only PEG treatment followed by ozone exposure significantly increased MDA ($P < 0.01$).

contained *lower* vitamin E concentrations than air-exposed skin (Table 1). The finding that covering the skin changed its antioxidant composition was unexpected. Most likely, covering the skin occluded it, increasing its moisture content, and changing its penetration characteristics [27]. Nonetheless, the skin vitamin E changes observed did not alter the conclusions of the study.

The ozone concentration (10 ppm) used in this study, given for a longer exposure time, causes lethal damage to the respiratory system. In urban air pollution, lower ozone concentrations (0.1–0.8 ppm) are encountered [4]. Since no other data concerning cutaneous effects of ozone exposure are available, the present study was designed to assess responses to high levels of ozone exposure. Further investigations are now needed to evaluate whether similar but less marked molecular skin damage takes place at lower, more relevant ozone concentrations and where this damage occurs. This most likely would require chronic or intermittent exposures and/or more sensitive techniques to differentiate and analyze the uppermost epidermal skin layers, in particular the stratum corneum.

In summary, the major finding of this study was that topically applied vitamin E forms are dramatically depleted by acute, short-term exposure to ozone. This is the first report showing that ozone is capable of initiating oxidative processes in cutaneous tissues. Whether ozone-related oxidative stress contributes to skin disorders as a result of lifetime exposure remains to be determined.

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References

[1] Office of Technology and Assessment (1989) U.S. Government Printing Office, Washington, DC.
[2] Lippmann, M. (1989) J. Abnorm. Psychol. 39, 672–695.
[3] Menzel, D.B. (1984) J. Toxicol. Environ. Health 13, 183–204.
[4] Mustafa, M.G. (1990) Free Radical Biol. Med. 9, 245–265.
[5] Pryor, W.A. and Church, D.F. (1991) Free Radical Biol. Med. 11, 41–46.
[6] Pryor, W.A. (1991) Am. J. Clin. Nutr. 53, 702–722.
[7] Cross, C.E., Motchnik, P.A., Bruener, B.A., Jones, D.A., Kaur, H., Ames, B.N. and Halliwell, B. (1992) FEBS Lett. 298, 269–272.
[8] Pryor, W.A., Squadrito, G.L. and Friedman, M. (1995) Free Radical Biol. Med. 19, 935–941.
[9] Traber, M.G. and Sies, H. (1996) Annu. Rev. Nutr. 16, 321–347.
[10] Serbinova, E., Kagan, V., Han, D. and Packer, L. (1991) Free Radical Bio.l Med. 10, 263–275.
[11] Cooney, R.W., France, A.A., Harwood, P.J., Hatch-Pigott, V., Custer, L.J. and Mordan, L.J. (1993) Proc. Natl. Acad. Sci. USA 90, 1771–1775.
[12] Elias, P.M. and Feingold, K.R. (1992) Semin. Dermatol. 11, 176–82.
[13] Shindo, Y., Witt, E., Han, D., Epstein, W. and Packer, L. (1994) J. Invest. Dermatol. 102, 122–124.
[14] Shindo, Y., Witt, E., Han, D. and Packer, L. (1994) J Invest. Dermatol. 102, 470–475.
[15] Shindo, Y., Witt, E., Han, D., Tzeng, B., Aziz, T., Nguyen, L. and Packer, L. (1994) Photoderm. Photoimmunol. Photomed. 10, 183–191.
[16] Fuchs, J., Mehlhorn, R.J. and Packer, L. (1989) J. Invest. Dermatol. 93, 633–640.
[17] Fuchs, J., Huflejt, M.E., Rothfuss, L.M., Wilson, D.S., Carcamo, G. and Packer, L. (1989) J. Invest. Dermatol. 93, 769–773.
[18] Podda, M., Weber, C., Traber, M. and Packer, L. (1996) J. Lipid Res. 37, 893–901.
[19] Weber, C., Podda, M., Rallis, M., Traber, M.G. and Packer, L. (1996) Free Radical Biol. Med. (in press).
[20] Dupuis, D., Rougier, A., Roguet, R., Lotte, C. and Kalopissis, G. (1984) J. Invest. Dermatol. 82, 353–356.
[21] Burton, G.W., Webb, A. and Ingold, K.U. (1985) Lipids 20, 29–39.
[22] Lang, J.K., Gohil, K. and Packer, L. (1986) Anal. Biochem. 157, 106–116.
[23] Podda, M., Weber, C., Traber, M.G. and Packer, L. (1996) J. Lipid Res. 37, 893–901.
[24] Shindo, Y., Witt, E. and Packer, L. (1993) J. Invest. Dermatol. 100, 260–265.
[25] Pryor, W.A. (1992) Free Radical Biol. Med. 12, 83–88.
[26] Frei, B., Stocker, R. and Ames, B.N. (1988) Proc. Natl. Acad. Sc.i USA 85, 9748–9752.
[27] Shaw, J.E., Prevo, M., R, G. and Yum, S.I. (1991) in: Physiology, Biochemistry, and Molecular Biology of the Skin (L.A. Goldsmith, Ed.) Oxford University Press, New York.