Ozone-Exposure Depletes Vitamin E and Induces Lipid Peroxidation in Murine Stratum Corneum

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The presence of ozone (O_3) in photochemical smog is an important health concern. We hypothesized that the stratum corneum (SC), as the outermost skin layer and the permeability barrier of the skin, represents a sensitive target for O_3-induced oxidative stress. To test this hypothesis, SKH-1 hairless mice were anesthetized and exposed for 2 h to O_3 by using two strategies: (i) single exposures to 0 (n = 12), 1 (n = 4), 5 (n = 4), and 10 (n = 4) ppm; and (ii) repeated daily exposures to 0 ppm (controls; n = 4) and 1 ppm (n = 4) for six consecutive days. New techniques based on the removal of SC by tape stripping were used to analyze the biologic effects of O_3 with respect to vitamin E depletion and lipid peroxidation. SC tissue was extracted from the tape and immediately analyzed by HPLC for vitamin E and malondialdehyde (MDA) concentrations. After *in vivo* exposure to increasing O_3 doses, vitamin E was depleted and MDA formation was increased, both in a dose-dependent manner. Remarkably, repeated low-level O_3 exposures resulted in cumulative oxidative effects in the SC: As compared with O_3 exposures of 0 ppm (α-tocopherol, 8.95 ± 1.3 pmol per mg; γ-tocopherol, 3.00 ± 0.3 pmol per mg; MDA, 3.69 ± 0.3 pmol per mg), vitamin E was depleted (α-tocopherol, 2.90 ± 0.6 pmol per mg, $p < 0.001$; γ-tocopherol, 0.5 ± 0.1 pmol per mg, $p < 0.001$) and MDA levels were increased (4.5 ± 0.2; $p < 0.01$). This report demonstrates the unique susceptibility of the SC to oxidative damage upon exposure to O_3. *Key words: tocopherol/anti-oxidants/skin/oxidative stress. J Invest Dermatol 108:753–757, 1997*

Ozone (O_3) in the upper atmosphere (stratosphere) occurs naturally and protects skin by filtering out solar ultraviolet radiation. O_3 at ground level (troposphere), however, is a noxious, highly reactive oxidant pollutant. It is the major component of photochemical smog and represents one of the most intractable urban air quality problems. In addition to photochemical smog, O_3 is generated during operation of high-voltage devices and dermatologic phototherapy equipment (Mustafa, 1990).

The skin is the organ most directly exposed to O_3. In fact, O_3 is probably the most reactive chemical to which the skin is routinely exposed in the environment. Numerous studies have documented the effects of O_3 on the respiratory tract in animals and humans (Menzel, 1984; Mustafa, 1990; Cross *et al,* 1994) and on plants (Hewitt *et al,* 1990). Therefore, we have undertaken studies investigating the effects of O_3 on anti-oxidants and lipids in skin.

In previous studies by our group, hairless mice were exposed to a single high dose of O_3 (10 ppm for 2 h). Measurements of anti-oxidants in whole skin failed to detect depletion of inherent anti-oxidants; however, malondialdehyde (MDA), a parameter of lipid peroxidation, was significantly increased. Furthermore, topically applied vitamin E was substantially depleted (Thiele *et al,* 1997a). On the basis of this work we hypothesized that the oxidative effects of O_3 in skin occurs mainly in the outer layers of skin. We therefore investigated the oxidative effects of a single dose of 10 ppm O_3 on three different layers of skin [(i) upper epidermis, (ii) lower epidermis and papillary dermis, and (iii) dermis). With this approach, we demonstrated that high O_3 levels significantly deplete inherent concentrations of vitamins C and E and induce MDA formation in the upper epidermis, including the stratum corneum (SC) (Thiele *et al,* 1997b).

Since we found the O_3 effects more pronounced in outer skin layers, we further hypothesized that the SC, the site of the air/tissue boundary, should be the most susceptible site for O_3-induced oxidative skin injury. Intriguingly, the SC is composed of a unique two-compartment system of structural enucleated cells (corneocytes) embedded in a lipid-enriched intercellular matrix (Elias and Feingold, 1992), forming stacks of bilayers that are rich in ceramides, cholesterol, and free fatty acids (Mao-Qiang *et al,* 1996). A major mechanism of O_3-induced tissue damage is thought to be the peroxidation of lipids, especially unsaturated fatty acids (Pryor and Church, 1991; Pryor *et al,* 1995). α-Tocopherol is generally regarded as the most important lipid soluble anti-oxidant (Traber and Sies, 1996) and inhibits O_3-induced lipid peroxidation in the respiratory tract (Mustafa, 1990; Pryor, 1991). This study was undertaken to test our hypothesis that the SC is a particularly susceptible site for O_3-induced oxidative stress and to investigate whether such effects occur at lower more environmentally relevant O_3 concentrations.

MATERIALS AND METHODS

Chemicals All chemicals used were of the highest grade available. Authentic α- and γ-tocopherol standards were a gift from the Henkel Corporation (La Grange, IL).

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Abbreviations: MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; SC, stratum corneum; TBA, thiobarbituric acid.

Animals The animal care, handling, and experimental procedures were carried out as described in the animal use protocol approved by Animal Care and Use Committee of the University of California, Berkeley, CA. Hairless mice (males, 7 wk old, Charles River Laboratories, Wilmington, MA) were kept under standard light and temperature conditions. Food (Harlan Teklad Rodent Diet 1846, WI) and water were provided *ad libitum*. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg per kg of body weight, Nembutal, Abbott Laboratories, North Chicago, IL) and remained anesthetized during the entire O_3- or air-exposure period.

Ozone Exposure O_3 was produced from oxygen by electric discharge (Sander ozonizer model IV, Eltze, Germany). The O_3 was then mixed with filtered (O_3 free) ambient air and allowed to flow into an exposure chamber at a constant rate (200 liters per min). The concentration in the exposure chamber was adjusted to 1, 5, and 10 ppm and continuously monitored with an O_3 detector (Dasibi model 1003-AH, Glendale, CA).

Single O_3 Exposure To evaluate possible dose-dependent effects of O_3, animals were exposed to various O_3 concentrations. Since the exposure chamber provided a maximum space for four animals, for each O_3 level (0, 1, 5, and 10 ppm), four animals were exposed; for the control level (0 ppm), 12 mice were exposed only to air, but treated identically in terms of anesthesia, housing, temperature, and light conditions.

Repeated O_3 Exposure To evaluate possible cumulative effects at a low O_3 level, four mice were anesthetized and exposed all at once to 1 ppm O_3 for 2 h, on six consecutive days. Four mice that were exposed to 0 ppm O_3, but treated identically in terms of anesthesia, housing, temperature, and light conditions, served as controls. The O_3-exposure chamber consisted of stainless steel and, thus, was impermeable for ultraviolet radiation during the O_3 exposure. To further rule out interfering effects with ultraviolet radiation after the O_3 treatment, the animals were kept in the dark until euthanized. In all cases, the animals were allowed to breathe air for 30 min, then SC was removed by tape stripping (see below), and the animals were sacrificed.

Tape Strippings Samples of murine SC were obtained by tape-stripping the skin with 5 cm × 5 cm pieces of cellophane tape. In preliminary studies, various tapes were tested for applicability and interferences with the high performance liquid chromatography (HPLC) methods for vitamin E and MDA analyses; the Scotch Superstrength Mailing Tape (3M, St. Paul, MN) was found to be the most suitable for the MDA assay, and the Highland 3710 (3M, St. Paul, MN) showed no interference with the HPLC analysis of tocopherols. We observed differences in interfering HPLC peaks between 3M 3710 tapes originating from different lots. It is therefore necessary to test for chromatogram interferences when first using a tape from a new lot.

To improve the reproducibility of the tape stripping technique, a standardized protocol was used: Tapes were cut exactly (5 × 5 cm), then smoothly adhered onto the animal's back, equally flattened three times, and gently removed by using moderate and even power of traction. The resultant SC layers adhered on the tapes appeared in the light microscope to be of homogenous thickness. The amount of SC was determined by the difference in weight before and after application and immediate removal of the skin. The amount of SC obtained per tape strip ranged from 2.6 to 3.4 mg and the mean weight was 2.98 ± 0.17 mg (±SD, n = 64). There were no differences in variations of weights obtained from different groups of animals (controls versus O_3 exposed).

Because of the possibility of surface contaminants, the first (uppermost) tape was discarded, the second tape was used for vitamin E analysis, and the third tape (on the same site) was used for MDA analysis. In preliminary experiments, no significant differences were found for either vitamin E or MDA measured in the second or third tape stripping after air or O_3 exposure (data not shown).

Tocopherol, Ubiquinol, and Ubiquinone Analysis After weighing, the tapes were transferred to a 50-ml polypropylene centrifuge tube (Corning Costar Corporation, Cambridge, MA) containing 2 ml of phosphate-buffered saline with 1 mM ethylenediamine tetraacetic acid, 50 μl of butylated hydroxytoluene (1 mg per ml), 1 ml of 2.9% (vol/vol) SDS, and 4 ml of ethanol. This was mixed vigorously and extracted with 4 ml of hexane. Ethanol (2 ml) was added to the hexane to precipitate the glue (originating from the tape adhesive), which was then removed with a spatula and discarded. The hexane/ethanol was taken to dryness under nitrogen; the residue was resuspended in 500 μl of ethanol:methanol (1:1). The sample then was injected into the HPLC system (Shimadzu, Kyoto, Japan), consisting of a SCL-10A system controller, a LC-10AD pump, and a SIL-10A autoinjector with sample cooler, an Ultrasphere ODS C_{18}, 4.6-mm internal diameter, 25 cm long, and 5-μm particle size column (Beckman, Fullerton, CA) with an All-Guard pre-column system (Alltech, Deerfield, IL), an in-line HP 1050 Diode Array Detector (Hewlett–Packard, Wilmington, DE), and a LC-4B amperometric electrochemical detector with a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN). The mobile phase was methanol:ethanol, 1:9 (vol/vol), with 20 mM lithium perchlorate. The flow rate was 1.2 ml per min. For measurement of ubiquinol, α- and γ-tocopherols, the electrochemical detector was operated with a 0.5-V potential and the full recorder scale at 50 nA. The diode array detector collected spectra at 275 nm for quantitation of ubiquinone 9. Data were collected with a Perkin–Elmer interface and analyzed by Turbochrom software (P.E. Nelson, Cupertino, CA).

Extraction Recovery Two aliquots containing equal amounts of SC obtained by tape stripping murine dorsal skin were used to determine anti-oxidant recoveries. Prior to the extraction, known amounts of α- and γ-tocopherol standards (dissolved in 100 μl of ethanol) were added to one aliquot, and 100 μl of ethanol were added to the other. Both samples were then extracted and analyzed as described above. The percent recovery was calculated by dividing the difference in amounts of internal standard obtained in the presence and absence of SC by the amount of added internal standard and multiplied by 100.

MDA A method was developed to detect lipid peroxidation in SC. Fluorimetric detection of the MDA-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 (Yu *et al*, 1986; Young and Trimble, 1991). After weighing, the adhesive tapes containing the SC were transferred to a test tube with 2 ml of methanol and 2 ml of SDS solution (0.15 g per 100 ml of HPLC grade water) and 100 μl of butylated hydroxytoluene (0.22 g per 100 ml of absolute ethanol). After mixing vigorously, 4 ml of chloroform were added and mixed again. This mixture was centrifuged for 7 min at 10,000 rpm, and then 3 ml of the lower layer (chloroform) were taken and mixed with 3 ml of ethanol to precipitate the adhesive, which was then removed and discarded. The remainder was dried down under N_2, and the residue was resuspended in 400 μl of SDS (0.15 g per 100 ml) and incubated with 250 μl of TBA reagent (0.375 g TBA per 100 ml) and 200 μl of phosphoric acid (1.22 M) for 30 min at 100°C. Then 380 μl of methanol were added for protein precipitation and 20 μl of 1 N NaOH was added for pH neutralization. After centrifugation, 100 μl of the clear supernatant was injected into the HPLC system, which consisted of a 114 M Solvent Delivery Module pump (Beckman, Fullerton, CA), a U6K Injector (Rheodyne, Cotati, CA) with a 100-μl sample loop, and an Alltima C_{18} 5-μm, 250 mm × 4.6 mm internal diameter, reversed-phase column with an All-Guard pre-column system (Alltech, Deerfield, IL), and a Hitachi (Hitachi, Tokyo, Japan) F-105 fluorescence spectrophotometer. The mobile phase consisted of 60% methanol and 40% 50 mM NaH_2PO_4, pH adjusted to 5.5. The flow rate was 0.9 ml per min and the detector was set at excitation 532 nm and emission 553 nm. MDA standards (ranging from 0.5 to 10 pmol) and samples using dilutions of 1,1,3,3-tetramethoxypropane were prepared under identical conditions. Samples and standards were analyzed in duplicate. Data were collected with a Perkin–Elmer interface and analyzed by Turbochrom software (P.E. Nelson, Cupertino, CA).

Statistical Analysis Statistical analysis was carried out with InStat 2.01 (GraphPad Software, San Diego, CA). Data were analyzed by one-way analysis of variance and the Tukey post test. The 0.05 level was selected as the point of minimal acceptable statistical significance. All data in text and figures are expressed as mean ± SD.

RESULTS

Tocopherol Measurement in SC Well-defined γ- and α-tocopherol peaks with retention times of 3.9 min and 4.1 min respectively were detected both in standards and SC samples (**Fig 1*a***). The identity of the peaks were confirmed by co-elution with authentic tocopherol standards and comparison of absorption spectra with those of tocopherol standards by using a diode array detector.

Electrochemical detector responses were linear from 0.5 to 100 pmol for each of the tocopherol standards. γ- and α-Tocopherol concentrations in SC were found within this range. The limit of detection was 0.1 pmol for both compounds. The percent recoveries of each of the tocopherols extracted from SC were evaluated as described. Recoveries were 64 ± 5% (mean ± SD) for α-tocopherol and 70 ± 4% for γ-tocopherol (mean ± SD). The results presented are not corrected for recovery and thus are a slight underestimation of the actual SC anti-oxidant contents. The vari-

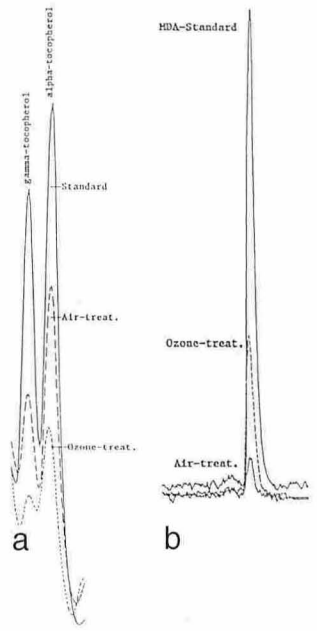

Figure 1. Vitamin E and MDA peaks from HPLC chromatograms. *(a)* Section of a representative HPLC chromatogram showing the γ- and α-tocopherol peaks in SC extracts. The peak height is proportional to the amount in the SC extract. Traces indicated are Standard (α-and γ-tocopherol standards), Air-treat. (SC taken after exposure of animals to air for 2 h), and Ozone-treat. (SC taken after exposure of animals to 5 ppm ozone for 2 h). *(b)* Section of a representative HPLC chromatogram showing malondialdehyde peaks in SC extracts. The peak height is proportional to the amount in the SC extract. Traces indicated are MDA standard, Air-treat. (SC taken after exposure of animals to air for 2 h), and Ozone-treat. (SC taken after exposure of animals to 5 ppm ozone for 2 h).

ances in vitamin E and MDA measurements in the SC were less than 10%.

Air Exposure (Controls) Vitamin E concentrations in the SC were measured in mice not exposed to O_3 (0 ppm, air-treat; **Fig 2**). The α- and γ-tocopherol concentrations were 8.4 ± 1.3 pmol per mg of tissue and 2.9 ± 0.9 pmol per mg of tissue, respectively (mean ± SD, n = 12 animals).

Single Ozone Exposure Exposure of mice to O_3 for 2 h depleted SC α-tocopherol concentrations in a dose-dependent manner **(Fig 2)**. α-Tocopherol concentrations after O_3 exposure to 1 ppm were 6.8 ± 0.5 pmol per mg of tissue ($p < 0.05$, as compared to animals exposed to 0 ppm O_3), to 5 ppm were 1.5 ± 0.4 pmol per mg of tissue ($p < 0.001$), and to 10 ppm were 0.7 ± 0.3 pmol per mg of tissue ($p < 0.001$). Similarly, γ-tocopherol concentrations were decreased with increasing O_3 exposure. γ-Tocopherol concentrations at 1 ppm were 2.1 ± 0.6 pmol per mg of tissue, at 5 ppm were 1.35 ± 0.7 pmol per mg of tissue ($p < 0.05$), and at 10 ppm were 0.4 ± 0.2 pmol per mg of tissue ($p < 0.001$). Each O_3 exposure represents means ± SD (n = 4 animals).

Repeated Ozone Exposures The SC tocopherol contents from mice that were exposed to 1 ppm O_3 for 2 h on six consecutive days were significantly depleted **(Fig 3)**. Specifically, the repeatedly exposed animals had markedly lower α-tocopherol (2.9 ± 0.6 pmol per mg, $p < 0.001$) and γ-tocopherol (0.5 ± 0.1 pmol per mg, $p < 0.001$) concentrations compared with mice exposed only to air (0 ppm O_3). These control mice had vitamin E concentrations (α-tocopherol, 8.95 ± 1.3 pmol per mg of tissue; γ-tocopherol, 3 ± 0.3 pmol per mg of tissue) similar to those animals exposed to air, which were described above.

MDA Measurement in SC A single well-defined peak with a retention time of 3.6 min corresponding to the MDA-TBA adduct was found in both standards and SC samples **(Fig 1*b*)**. The identity of the peak was confirmed by co-elution with MDA standards and by comparison of its absorption spectrum with that of authentic MDA standards by using a diode array detector. Fluorescence detector responses were linear from 0.5 pmol to 10 pmol for the MDA standards. All SC samples measured were within this range. The detection limit was 0.5 pmol.

Single Ozone Exposure Induces Dose-Dependent Formation of MDA SC MDA levels in animals exposed to air (0 ppm O_3) for 2 h were 3.3 ± 0.4 pmol per mg of tissue. Exposure to O_3 at 1 ppm for 2 h did not increase MDA concentrations (3.8 ± 0.2 pmol per mg of tissue; **Fig 2**). MDA levels, however, were significantly increased after exposure to 5 ppm O_3 (5.3 ± 0.5 pmol per mg of tissue; $p < 0.001$) or 10 ppm O_3 (8.6 ± 0.6 pmol per mg of tissue; $p < 0.001$).

Repeated Ozone Exposures Induce Cumulative MDA Formation Repeated exposure to 1 ppm O_3 resulted in significantly higher MDA concentrations (4.5 ± 0.2; $p < 0.01$; **Fig 3**) compared to exposure to 0 ppm O_3 (3.69 ± 0.3 pmol of mg of tissue). These latter values were not different from those in animals anesthetized and exposed to air, as described above.

DISCUSSION

This report documents concentrations of α- and γ-tocopherols and MDA in the SC. We demonstrate that O_3 depletes vitamin E in a dose-dependent fashion (0–10 ppm) and induces lipid peroxidation in the SC. Most notably, repeated exposures to the lowest tested O_3 level (1 ppm) exerted cumulative oxidative stress effects. The methods reported herein are based on tape stripping and subsequent HPLC analysis of SC extracts. They enabled us to sensitively (detection limit in the pmole range) and specifically measure vitamin E (α- and γ-tocopherols) and MDA (in contrast to the most

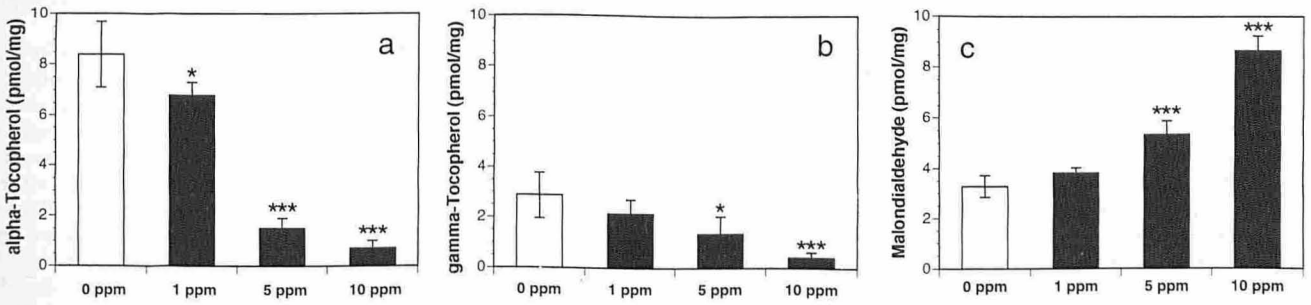

Figure 2. Ozone depletes vitamin E and induces MDA formation in the SC in a dose-dependent manner. *(a)* α-Tocopherol concentrations in SC extracts. *(b)* γ-Tocopherol concentrations in SC extracts. *(c)* MDA concentrations in SC extracts. The *x* axis gives the ozone concentration in the atmosphere of exposure chamber. Controls, 0 ppm ozone (*shaded bar*, n = 12); ozone exposed, 1, 5, 10 ppm (*solid bars*, n = 4 for each group). $^{*}p < 0.05$; $^{***}p < 0.001$. *Error bars*, SD (n = 4).

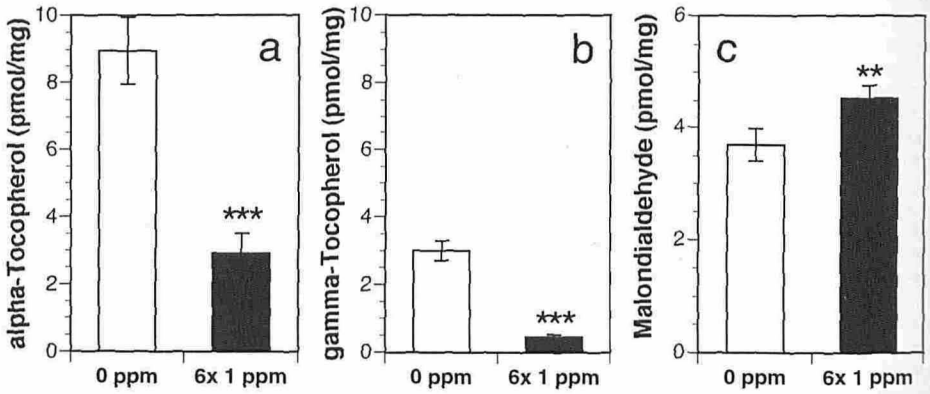

Figure 3. Repeated low-dose ozone exposure induces cumulative vitamin E depletion and MDA formation in the SC. *(a)* α-Tocopherol concentrations in SC extracts. *(b)* γ-Tocopherol concentrations in SC extracts. *(c)* MDA concentrations in SC extracts. The *x* axis gives the ozone concentration in atmosphere of exposure chamber. Controls, 0 ppm ozone; 6× 1, repeated 2-h exposures to 1 ppm ozone on six consecutive days. $^{**}p < 0.01$; $^{***}p < 0.001$. *Error bars*, SD (n = 4).

frequently measured, but nonspecific thiobarbituric acid reactive substances) in SC **(Fig 1)**.

The high susceptibility of vitamin E in the SC upon O_3 exposure **(Figs 2 and 3)** indicates the potential for environmental O_3 to affect anti-oxidant defense mechanisms in cutaneous tissues. In most biologic systems, the depletion of co-anti-oxidants such as ascorbate or ubiquinol, which serve to regenerate vitamin E (Kagan *et al,* 1992; Stoyanovsky *et al,* 1995), precedes the depletion of the latter (Frei *et al,* 1988; Esterbauer *et al,* 1991). No detectable amounts of coenzyme Q (ubiquinol and ubiquinone) were found in the SC [detection limit, 0.1 pmol]. This is not surprising, since the density of mitochondria, the major site of coenzyme Q, decreases from the inner epidermal layers to the outer, and no mitochondrial activity is found in the SC (Braun-Falco, 1961). Coenzyme Q protects vitamin E from free radical attack and prevents it from depletion (Stoyanovsky *et al,* 1995). This lack of co-anti-oxidant may contribute to the significant loss of α-tocopherol observed in SC exposed to O_3.

It is known from *in vitro* experiments that O_3 reacts with polyunsaturated fatty acids (PUFAs) to form free radicals and that vitamin E slows or prevents this reaction (Pryor, 1991). In principle, the protection that vitamin E provides to PUFAs could arise by two different mechanisms: In the first, vitamin E could react directly with O_3 in a sacrificial way to destroy the O_3. In the second mechanism, O_3 could react with PUFAs to form radicals, which initiate autoxidation of the PUFAs, and vitamin E could act as scavenger of PUFA-derived radicals, stopping the autoxidation.

Most notably, MDA concentrations in the SC were dramatically increased after O_3 exposure **(Figs 2*c* and 3*c*)**. Since the first tape stripping, which contains the major part of the surface lipids, was discarded, and MDA was measured in the third tape stripping on the same site, it seems very unlikely that the MDA data is largely influenced by lipid peroxidation of surface lipids. The increase of the lipid peroxidation product MDA more likely reflects a significant amount of oxidative lipid alterations in the SC. MDA can be produced from direct ozonation of PUFAs by at least two mechanisms (Pryor, 1991): (i) MDA could result from the reaction of two O_3 molecules with a single PUFA molecule that has at least two methylene-interrupted double bonds; vitamin E does not inhibit this process (Roehm *et al,* 1971). (ii) MDA can also be produced during the O_3-initiated autoxidation of PUFAs that have at least three double bonds. This mechanism for MDA production from a trienoic acid, such as linolenate, involves the cyclization of the PUFA peroxyl radical to yield a prostaglandin-like endoperoxide, which hydrolyzes to yield MDA; this process could be inhibited by vitamin E (Pryor *et al,* 1976).

O_3-induced lipid peroxidation in the SC may be harmful to skin in two ways. (i) Oxidation and degradation of SC lipids could affect the barrier function of the SC. It is generally accepted that SC lipids play an important role for barrier integrity (Elias, 1983; Bouwstra *et al,* 1996). Perturbations of the SC lipid and protein architecture have been suggested to be an important trigger factor for a number of dermatoses (e.g., psoriasis, atopic dermatitis, and irritant dermatitis) rather than the end result of processes that are initiated in subjacent skin layers (Elias and Feingold, 1992; Mao-Qiang *et al,* 1996). The increase in the lipid peroxidation product MDA in the SC measured after O_3 exposure involves peroxidation of PUFAs, such as arachidonic acid and linolenate. This process reflects significant changes in the lipid composition of the SC, which could conceivably affect epidermal function, even though the SC consists of enucleated "dead" cells.

(ii) The increased formation of lipid peroxidation products in upper skin layers could trigger an inflammatory response in adjacent skin layers. *In vivo*, O_3 toxicity is believed to result from the effects of a cascade of products that are produced in the reactions of O_3 with primary target molecules that lie close to the air/tissue interface (Pryor *et al,* 1992; Pryor *et al,* 1995; Uppu *et al,* 1995). O_3 itself is generally believed to be too reactive to penetrate far into tissue; only a small fraction of environmentally relevant doses of O_3 are believed to pass unreacted through a bilayer membrane, and none may pass through a cell (Pryor, 1992). Secondary (or tertiary) O_3-induced lipid peroxidation products, which have a lower reactivity and longer lifetime than O_3 itself, transmit the effects of O_3 beyond the air/tissue interface (Pryor, 1992). Due to their relative stability, lipid peroxidation products (e.g., 4-hydroxy-alkenals and aldehydes, such as MDA) can damage or alter cells and tissues at more distant sites not directly exposed to O_3 (Pryor *et al,* 1995). Similarly, there exists evidence that ultraviolet-light-induced skin damage is mediated, in part, by secondary products of oxidative processes (Gilchrest, 1995; Pentland, 1996).

The formation of O_3 in the troposphere requires the presence of ultraviolet radiation, a known inducer of oxidative stress in skin (Chen *et al,* 1996). Since the O_3 exposure carried out in this study was performed in the dark in a stainless steel chamber and the animals were kept in the dark until euthanized, the observed oxidative stress effects were not influenced by UV irradiation. In urban pollution, however, the concomitant exposure to O_3 and ultraviolet radiation in photochemical smog could cause synergistic oxidative stress effects in skin.

The inverse relationship evaluated in this study for vitamin E and MDA concentrations in SC after exposures to increasing O_3 concentrations **(Fig 2)** suggests a key role for this anti-oxidant in the prevention of oxidative damage in the SC. Furthermore, vitamin E has been shown to act as a penetration enhancer by intercalating within the lipid bilayer region of human SC, altering the characteristics of the membrane affecting permeability (Trivedi *et al,* 1995). Thus, both vitamin E content and lipid composition play major roles in permeability barrier homeostasis of the SC. Interestingly, α-tocopherol appears to be more readily depleted by O_3 than γ-tocopherol, suggesting a higher anti-oxidant activity for the former. This observation is in accordance with findings in other tissues (Kamal-Eldin and Appelqvist, 1996).

The O_3 concentrations used in the present study were 1, 5, and 10 ppm for 2 h. It should be noted that given longer exposure

times, the exposures of 5 and 10 ppm cause lethal damage to the respiratory system. In urban air pollution, much lower O_3 concentrations (0.1–0.8 ppm) are encountered (Mustafa, 1990). The single 2-h exposure to 1 ppm O_3 resulted in SC α-tocopherol (not γ-tocopherol) depletion but not in increased SC lipid peroxidation as measured by MDA **(Fig 2)**. Most remarkably, however, we found significant vitamin E depletion and MDA increase when exposing the animals to 1 ppm repeatedly **(Fig 3)**, suggesting cumulative oxidative stress effects. Thus, O_3-mediated oxidative damage of the SC may occur at lower O_3 concentrations, which approach those levels encountered in urban centers for many days per year (U.S. Environmental Protection Agency, 1993).

We report the measurement of vitamin E (α- and γ-tocopherol) and MDA in the SC. We demonstrate that upon *in vivo* exposure to O_3, SC vitamin E is depleted and MDA formed in a dose-dependent manner and that this occurs at O_3 levels approaching those found in photochemical smog. These findings suggest that the SC could serve as an intrinsic dosimeter for assessing environmental oxidative damage such as caused by O_3. Furthermore, our findings may have implications for the pathophysiology of skin disorders that reportedly occur with increasing frequency in air-polluted urban areas, such as atopic dermatitis (Schultz-Larsen, 1993). Further studies are required to investigate qualitative and quantitative changes in the SC-lipid composition occurring during O_3 exposure, as well as their impact on the epidermal barrier function.

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