COMMUNICATION

Vitamin C, Uric Acid, and Glutathione Gradients in Murine Stratum Corneum and their Susceptibility to Ozone Exposure

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The stratum corneum has been recognized as the main cutaneous oxidation target of atmospheric ozone (O_3), a major part of photochemical smog. This study reports the presence and distribution of vitamin C, glutathione, and uric acid in murine stratum corneum, and evaluates their susceptibility to acute environmental exposure to O_3. Based on tape stripping and a modified extraction method with high performance liquid chromatography electrochemical analysis, we detected vitamin C (208.0 ± 82.5 pmol per 10 consecutive pooled tapes), glutathione (283.7 ± 96.3), and uric acid (286.4 ± 47.1) in murine stratum corneum as compared with only 16.5 ± 1.4 pmol α-tocopherol. Vitamin C, glutathione (both $p < 00.001$), and urate ($p < 0.01$) were found to exhibit a gradient with the lowest concentrations in the outer layers and a steep increase in the deeper layers. To investigate the effect of O_3 exposure on hydrophilic antioxidants, we exposed SKH-1 hairless mice to O_3 concentrations of 0, 0.8, 1, and 10 p.p.m., and stratum corneum was analyzed before and after exposure. Whereas mock exposure with 0 p.p.m. for 2 h had no significant effect, O_3 doses of 1 p.p.m. for 2 h and above showed depletion of all three antioxidants. Vitamin C was decreased to 80% ± 15% of its pretreatment content ($p < 0.05$), GSH to 41% ± 24% ($p < 0.01$), and uric acid to 44% ± 28% ($p < 0.01$). This report demonstrates the previously unrecognized role of hydrophilic antioxidants in the stratum corneum and provides further evidence that O_3 induces oxidative stress in this outer skin layer. ***Key words: antioxidant/oxidative stress/skin/skin barrier/vitamin E. J Invest Dermatol 113:1128–1132, 1999***

Ozone (O_3) is a double-edged molecule. On the one hand, it plays a major role in filtering out the short-wave spectrum of solar UV radiation in the stratosphere. On the other hand, it can be noxious at ground level (troposphere), where it is an important environmental pollutant, and rapidly reacts with a variety of target molecules (Mustafa, 1990). The cutaneous tissues, along with the respiratory tract, are the most directly exposed organs to tropospheric O_3; however, very little is known about O_3-toxicity in the skin.

Previous studies by our group have investigated its effect on antioxidants in murine skin (Thiele *et al*, 1997a, b). These studies showed that O_3 exposure caused significant vitamin E and C depletion as well as lipid peroxidation at a dose of 10 p.p.m. for 2 h. The stratum corneum (SC) represented the major target for O_3, which is not surprising as the tissue/air interface is the first part of the skin to come in contact with O_3. SC α-tocopherol was a susceptible target to O_3 exposure at the much lower doses used before. Even a single exposure of 1 p.p.m. for 2 h significantly decreased vitamin E in murine SC. Taking into account that the maximum levels of tropopspheric O_3 in heavily polluted urban areas is 0.8 p.p.m. (Mustafa, 1990), the effects were thus seen at doses close to those that could be encountered under these conditions. Importantly, repeated exposure to the same dose resulted in further depletion of vitamin E as well as in lipid peroxidation (Thiele *et al*, 1997a).

A paucity of studies exist concerning other antioxidants in the SC. Total thiols were estimated to be 60% of the content in epidermis (Flesh and Satanoce, 1955), and vitamin C was 17% of the concentration in whole skin (Patscher and Roeckl, 1960). To our knowledge the SC levels of glutathione (GSH) and uric acid, two effective hydrophilic antioxidants (Ames *et al*, 1981), have not been characterized. This study was carried out to investigate the presence and distribution of GSH, uric acid, and vitamin C in sequential layers of murine SC and their susceptibility to depletion by O_3.

MATERIALS AND METHODS

Chemicals All chemicals were of the highest grade available. Ascorbic acid, urate, and glutathione standards were purchased from Sigma (St. Louis, MO).

Rationale for the use of animals and procedures SKH1-female hairless mice between 6 and 9 wk old (Charles River Laboratories, Wilmington, MA) were used for this study. Although it would have been preferable to have studied humans, we wanted to first investigate the murine model to obtain basic information on O_3-induced damage, so that human experiments can be carried out safely as the next step. The SKH-1 mouse model is widely used to simulate human skin in toxicologic studies (Kligman and Kligman, 1998). The lipophilic antioxidant vitamin E was shown to be distributed similarly in SC of both the SKH-1 hairless mouse

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Abbreviations: AU, arbitrary unit; GSH, glutathione; O_3, ozone; SC, stratum corneum.

model and the human skin, and to be depleted similarly by ultraviolet radiation (Thiele *et al*, 1998a). The SC of this mouse has been extensively used to study skin barrier properties (Elias and Feingold, 1992). Mice were housed under standard conditions (12 h light dark circle at 22°C and 55% humidity). Feed (Harlan Teklad Rodent Diet #1846, WI) and tap water were provided *ad libitum*. The 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. For experiments, the mice were anesthetized with sodium pentobarbital.

Ozone exposure O_3 was generated from oxygen by electrical discharge (Sander ozonizer model IV, Eltze, Germany) and mixed with filtered ambient air as described earlier (Thiele *et al*, 1997a). The exposure chamber was provided with a constant flow rate of 200 liters per min. O_3 levels were adjusted between 0, 0.8, 1, and 10 p.p.m. and were continuously monitored with an O_3 detector (Dasibi model 1003-AH, Glendal, CA). The animals were exposed in groups of two for 2 h each (n = 6 per group).

Tape stripping of stratum corneum To measure antioxidant gradients, sequential tape stripping on two dorsal locations of the anesthetized animal was performed (D-Squame-tapes, Cuderm, Dallas, TX). A total of 10 strips for each location was applied uniformly and removed gently in a standardized manner, as described earlier (Thiele *et al*, 1998a). Each of the sequential tapes was assigned a number between 1 and 10. Tapes 1–2, 3–4 and so on, were grouped and pooled with the corresponding layers of the contralateral location. In this way, five consecutive layers of four tapes each were formed. To protect the antioxidants, the strips were stored at –80°C in the dark until analysis for not more than 30 min. The average weight of a tape strip (surface area 3.80 cm^2) was determined to be 30 ± 9 μg per cm^2 (n = 25). As described before (Thiele *et al*, 1998a), the thickness of the SC removed from 10 tape strips was roughly estimated to be 3.5 μm, assuming a uniform coverage of SC on the tape strip and a density of 1 g per cm^3. The protein concentration in tape strips has been shown to be a closely correlating indicator of the amount of SC removed by tape stripping (Dreher *et al*, 1998). The protein content in the hydrophilic extracts of the pooled tape strips was analyzed using the BCA-Protein Assay (Pierce, Rockford, IL). The protein content per four pooled tape strips was 3.76 ± 1.18 μg in the first layer, remained relatively constant in the next two layers, and showed a trend to decrease in layer 4 (2.98 ± 1.23 μg) and layer 5 (2.29 ± 1.64 μg) (**Fig 2*a***), which failed to reach significance within the sample size. This is an indication that the mass of the SC removed showed a decreasing trend in the two deepest layers analyzed. As a consequence, the depth of SC was displayed as the distinct categories layers 1–5 without assuming linearity. For O_3-exposure experiments, one side of each animal was tape stripped prior to exposure, and the contralateral side 20 min after exposure. A total pool of six tapes was obtained for each spot.

Analysis of ascorbic acid, uric acid, glutathione, and vitamin E The samples were extracted in 940 μl ice cold, chelexed phosphate-buffered saline containing 1 mM ethylenediamine tetraacetic acid and 60 μl butylated hydroxytoluene (10 mg per ml), by vigorous vortexing for 2 min. After filtering, the supernate was immediately injected into a high performance liquid chromatography system consisting of a LC-10AD pump (Shimadzu, Kyoto, Japan), a Microsorb -MV C18, 12 cm, 3 μm, 100 A column (Rainin, Woburn, MA) connected to an LC-4B amperometric detector, set at 500 mV. Uric acid and ascorbic acid standards were prepared daily as described (Thiele *et al*, 1997b). The compounds were eluted in a mobile phase consisting of 40 mM sodium acetate with 0.537 mM EDTA, 5% methanol, and 0.3% Q12 ion pair cocktail (Regis, Morton Grove, IL).

Glutathione was detected according to a modified method described elsewhere (Allison and Shoup, 1983). An aliquot of the same extract was eluted from an Altima C18 250 mm, 4.6 I.D., 5 μM column (Alltech, Deerfield, IL) with 100 mM monochloroacetic acid (pH 3–3.5) containing 4% methanol and detected with a thiol-selective gold mercury electrode.

To compare the levels of hydrophilic antioxidants to levels of vitamin E under the experimental conditions of this study, tape stripping layers 1–5 were analyzed in three mice. α-Tocopherol was extracted and separated on a reversed phase C18 column coupled to an amperometric detector as described (Thiele *et al*, 1998a).

Statistical analysis One-factor, repeated measure ANOVA with Tukey post test (InStat, Graph Pad Software, San Diego, CA) was applied; either repeated measure for related data sets or single measure for interindividual comparisons. All data was normalized with protein concentrations or expressed per tape strip. For analysis of the O_3 effect between different treatment groups, the ratios of antioxidant concentrations after and before treatment were used. Thus, each mouse served as its own control. The data are expressed as the remaining amount (%) after treatment (treated × 100/ untreated). A p value of < 0.05 was considered statistically significant. All data are expressed as mean ± standard deviation.

RESULTS

GSH and uric acid are present in murine SC GSH and urate were found in SC. In a total of 10 consecutive tapes, we detected 286.4 ± 47.1 pmol urate, 283.7 ± 96.3 pmol GSH, and 208.0 ± 82.5 pmol ascorbate (no significant difference), as compared with only 16.5 ± 1.4 pmol of α-tocopherol ($p < 0.01$ *versus* urate and GSH) (**Fig 1*a***). If only the first six consecutive tape strips were pooled, the values shifted significantly ($p < 0.0001$) to 63.8 ± 9.8 pmol for urate (corresponding to 22% of the amount in 10 strips), 44.2 ± 10.3 pmol for GSH (15%), 19.9 ± 8.3 pmol for ascorbate (9%), and 7.4 ± 0.6 pmol for α-tocopherol ($p < 0.01$ *versus* urate and GSH, $p < 0.05$ *versus* ascorbate) (**Fig 1*b***).

Vitamin C, GSH, and uric acid form gradients in murine SC Vitamin C concentrations per protein (pmol per μg protein) increased from the outside to the inside, forming a steep gradient (**Fig 2*a***). The range was from 8.6 ± 6.3 pmol per mg (protein) in layer 1 to 184.0 ± 68.2 pmol per mg in layer 5, a 21.4-fold increase, with the first significant difference in layer 4 as compared with layer 1 ($p < 0.001$). A similar distribution was observed for GSH (**Fig 2*b***). Layer 1 contained 16.6 ± 0.3 pmol per μg (protein), whereas layer 5 contained 123.4 ± 56.2 pmol per μg, here a 7.4-fold increase. Significant differences were reached first between layers 1 and 4 ($p < 0.001$). The urate content also increased towards deeper layers. Concentrations of urate ranged from 36.4 ± 3.7 pmol per mg (protein) in the uppermost to 136.0 ± 37.2 pmol per mg in the deepest layer. Starting from layer 3, the tapes contained significantly more urate then the first layer ($p < 0.01$); however, the distribution of urate was different from those of vitamin C and GSH. In the first three layers (the upper three layers) we found more urate than either GSH or vitamin C (layers 1 and 2, $p < 0.001$; layer 3, $p < 0.05$). In addition, layers 1 and 2 also contained more GSH than vitamin C ($p < 0.05$).

Uric acid, vitamin C, and GSH in SC are depleted by ozone As a mock treatment, one group (n = 6) was exposed to

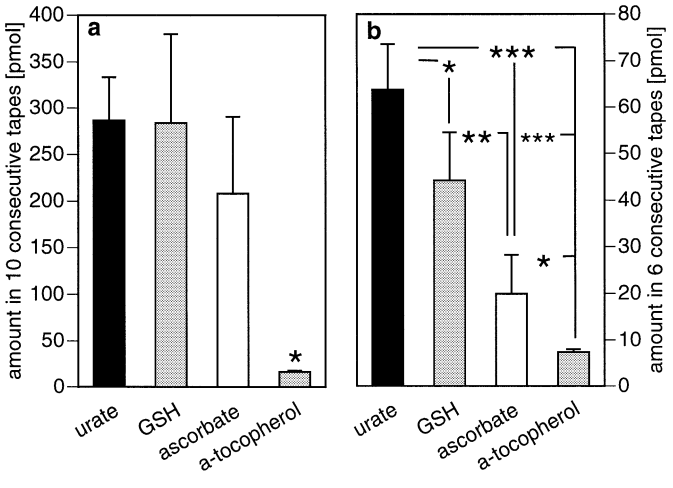

Figure 1. Murine SC contains hydrophilic low molecular weight antioxidants in higher amounts than vitamin E. (*a*) Cumulative amount of urate, GSH, ascorbate, and α-tocopherol in 10 consecutive tape strips, which corresponds to an area of 40 cm^2 and an estimated depth of 3.5 μm (n = 4 for urate, GSH, ascorbate, n = 3 for α-tocopherol) (*$p < 0.05$ *versus* ascorbate and $p < 0.01$ *versus* others). (*b*) Cumulative amount of the four compounds in the upper six tape strips. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The ratios were 3.2:2.2:1:0.4 (urate:GSH:ascorbate:α-tocopherol).

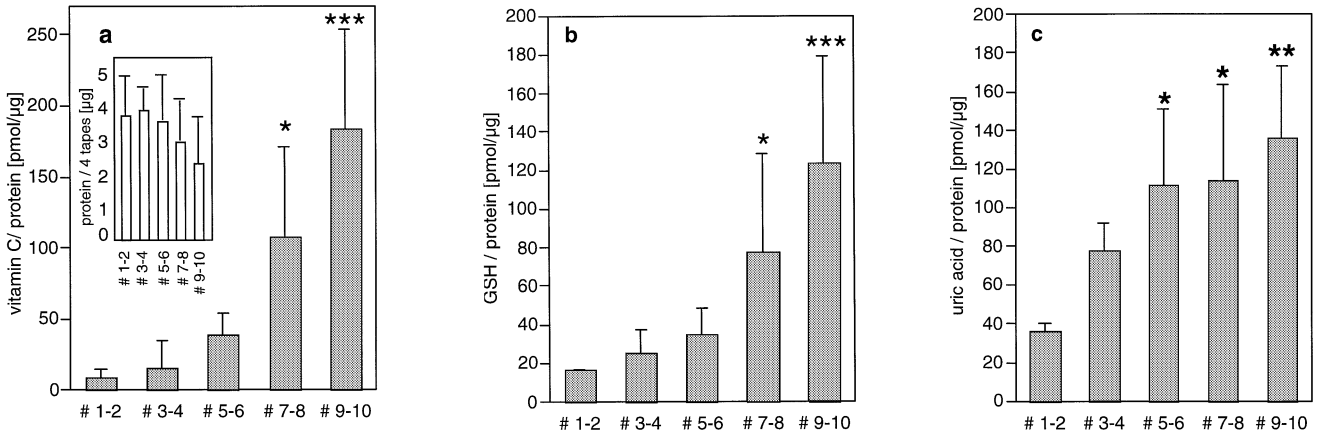

Figure 2. Ascorbate, GSH, and urate form gradients in SC. SC was sequentially stripped 10 times and each two consecutive strips pooled forming five layers (n = 4). Tapes 1–2 formed the most superficial layer. Layer 1 was compared with the subsequent layers (*$p < 0.5$, **$p < 0.1$, ***$p < 0.01$ as indicated in graphs). (*a*) Amount of vitamin C per protein as a function of SC layer. Statistical significance was first reached in tapes 7–8 ($p < 0.05$). The insert displays the distribution of protein in the different layers of tape strippings, displaying a not significant trend towards decreasing protein contents in the two deepest layers (see also *Materials and Methods*). (*b*) GSH per protein, first significant difference in layer 4 ($p < 0.05$). (*c*) Urate per protein, first significant difference in tapes 5–6 (*$p < 0.05$).

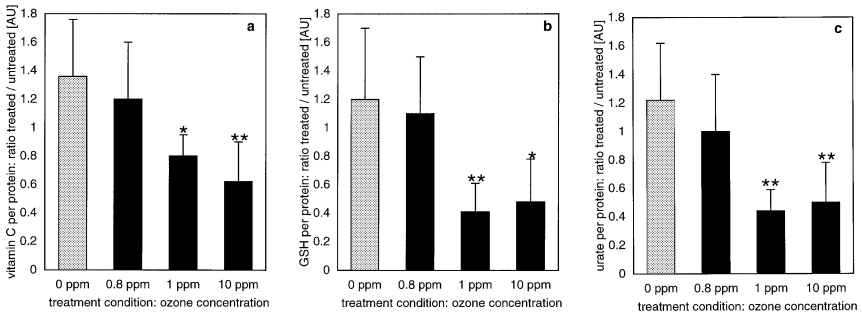

Figure 3. Hydrophilic antioxidants are depleted by O_3. Mice were exposed to O_3 for 2 h (n = 6 per treatment group) and tape stripped (six consecutive strips pooled) before and after treatment. The amount of SC antioxidant per protein after treatment in per cent of pretreatment values was plotted as a function of O_3 concentration. (*a*) Vitamin C, first significant difference at 1 p.p.m. (*$p < 0.05$); (*b*) GSH, first significant difference at 1 p.p.m. (**$p < 0.01$); (*c*) uric acid, first significant difference at 1 p.p.m. (**$p < 0.01$).

air only ("0 p.p.m."), without detecting significant depletion. In fact, air only exhibited a trend to increase the antioxidant concentration/protein.

Ascorbic acid The effect of O_3 treatment on SC ascorbic acid was significant ($p < 0.01$) (**Fig 3*a***), but less pronounced than in the case of uric acid and GSH. Whereas treatment with 0.8 p.p.m. (remaining amount after treatment 121% ± 40%) showed a slight trend towards a decrease as compared with 0 p.p.m. (136% ± 42%), statistical significance was reached at 1 p.p.m. ozone (80% ± 15%) ($p < 0.05$). 10 p.p.m. ozone depleted vitamin C to 62% ± 28% of the initial value ($p < 0.01$).

GSH Ozone treatment clearly depleted GSH in murine SC ($p < 0.01$) (**Fig 3*b***). At the 1 p.p.m. level, only 41% ± 24% of the pretreatment content remained ($p < 0.01$). Also, treatment with 10 p.p.m. exhibited a significant effect (48% ± 18%) ($p < 0.05$). At 0.8 p.p.m., the decrease was not significant (108% ± 43% remaining as compared with 115% ± 52% for 0 p.p.m.).

Uric acid O_3 had a very significant effect on uric acid concentrations in SC ($p < 0.001$) (**Fig 3*c***). 1 p.p.m. O_3 decreased uric acid to 44% ± 28% of the value before treatment, and after exposure to 10 p.p.m. 51% ± 25% remained. Thus, 10 p.p.m. were not more effective than 1 p.p.m. Exposure to 0.8 p.p.m. showed a trend to deplete uric acid (100% ± 22% remaining as opposed to 122 ± 50% for 0 p.p.m. treatment); however, this effect did not reach statistical significance within the sample size.

DISCUSSION

This study reports the presence and distribution of the major small molecular mass antioxidants urate, GSH, and vitamin C, in murine SC. It is demonstrated that hydrophilic antioxidants are in fact more abundant in the analyzed portion of the SC than vitamin E, and that urate exhibited the highest levels among the hydrophilic antioxidants in the upper SC. These findings are in accordance with the distribution pattern of antioxidants in the epidermis, the plasma, and the bronchoalveolar lining fluid (Shindo *et al*, 1993; Van der Vliet *et al*, 1999). At levels as low as 1 p.p.m. × 2 h, the susceptibility of these three antioxidants to O_3 exposure is demonstrated. Because the concentrations of the compounds are lower in SC than in the epidermis (Shindo *et al*, 1993), it must be assumed that they are partially consumed during the terminal differentiation of keratinocytes, perhaps due to exposure to ambient ultraviolet-radiation and/or exposure to successively higher levels of ambient O_2.

Urate, ascorbate, and GSH were all decreased from deeper layers towards the upper layers of the SC. Recently, a similar pattern was described for the distribution of vitamin E in human SC (Thiele *et al*, 1998a). The most probable explanation for these gradients is a gradual oxidation of the antioxidants during chronic exposure to the environment as the corneocytes egress toward the surface. The lack of protective hydrophilic antioxidants in the upper SC may explain the existence of a recently described physiologic keratin 10 oxidation gradient in SC, with 3-fold higher protein carbonyl concentrations in the upper SC as compared with deeper layers (Thiele *et al*, 1999). This protein oxidation gradient is speculated to be involved in the desquamation process, because oxidatively modified proteins are more susceptible to proteases. Thus, the gradients for hydrophilic antioxidants described in this paper may be indirectly involved in the desquamation process.

Ascorbate and GSH are relatively more depleted in the upper SC when compared with urate. This may be explained by possible recycling mechanisms of oxidized antioxidants.

Due to its higher redox, urate is unable to recycle the radicals of vitamin E, ascorbate or GSH. On the other hand, vitamin C can recycle the tocopheryl radical (Buettner 1993) in several systems, including the skin (Kagan *et al*, 1992), and even regenerates the urate radical (Maples and Mason, 1988). For example, it has been shown that plasma ascorbate is depleted in conditions of oxidative stress, before significant urate depletion occurs (Frei *et al*, 1988). Under slightly acidic conditions, which are present in the SC (Ohman and Vahlquist, 1994), GSH is able to regenerate ascorbate from dehydroascorbate nonenzymatically, which leads to loss of GSH (Stocker *et al*, 1986).

The interactions discussed are all based on the assumption that the antioxidants are homogeneously distributed in adjacent hydrophilic and lipophilic compartments. Because SC represents a complex structure with protein-rich corneocytes and lipid-rich intercellular domains, it is impossible to anatomically localize the analyzed compounds in SC substructures by tape stripping. The three hydrophilic antioxidants are probably located close to the core proteins in the small hydrophilic compartment (water content of SC only 15%, Mak *et al*, 1991). An alternative location could be the *lacunar* domains, which may serve as a hydrophilic pathway through the SC (Menon and Elias, 1997).

The depletion of urate, ascorbate, and GSH by O_3 can be due to the scavenging of free radicals as well as to the direct reaction with O_3 (Pryor *et al*, 1995). In this study, uric acid and GSH were more susceptible than ascorbic acid, which may be due to the fact that most of the O_3 was probably already reacted in the upper SC, where urate is the most abundant antioxidant target. This order was also observed in studies of respiratory tract lining fluid models (Mudway and Kelly, 1998), where ascorbic acid and uric acid were both rapidly consumed by O_3 in plasma (Cross *et al*, 1992).

In which ways could O_3 be harmful to the skin? (i) Oxidative damage by O_3 might affect the structural integrity of the SC by compromising antioxidant defences and inducing oxidative damage to lipids (Uppu *et al*, 1995; Thiele *et al*, 1998a) and proteins (Thiele *et al*, 1998b). This could lead to barrier perturbation, a pathophysiologic mechanism that has been shown to be involved in the pathogenesis of several skin pathologies (e.g., psoriasis, atopic dermatitis, irritant contact dermatitis, and aging skin including puritus of aging skin) (Elias and Feingold, 1992; Ghadially *et al*, 1995; Mao-Qiang *et al*, 1996). The fact that a dose of 10 p.p.m. $\times$ 2 h O_3 failed to decrease the levels of antioxidants more than 1 p.p.m. $\times$ 2 h may be an indirect indication of a disturbed barrier. With increased transepidermal water flux aqueous antioxidants could ''leak'' into the SC and mask the effects of higher O_3 doses. (ii) In the lung, it was proposed that not O_3 itself, but secondary ozonation products including alkanals are the mediators of O_3 toxicity (Pryor *et al*, 1995; Postlethwait *et al*, 1998). In analogy to the mechanisms described in the lung, a similar mode of O_3 action may occur in the skin. Aldehydes from unsaturated fatty acids, e.g., hexanal generated from linoleic acid (Postlethwait *et al*, 1998), could surpass the skin barrier and elicit toxic effects like inflammation or cell death in deeper layers of the skin. In fact, the formation of cells with shrunken nuclei was observed in a stratified human keratinocyte culture.[1]

In conclusion, this study emphasizes the formerly unrecognized role of hydrophilic antioxidants in the SC. It further clearly demonstrates that not only ultraviolet radiation, but also gaseous oxidants such as O_3, are able to induce oxidative stress in the skin. Significant depletion occurred at 1 p.p.m., a concentration slightly higher than the highest recorded in the environment (0.8 p.p.m.) (Mustafa, 1990). The overall effect of gaseous stressors may be much greater than the effect seen by O_3 alone, because other air pollutants such as nitric oxides and volatile reactive oxygen species (Mustafa, 1990) may work in a similar fashion as O_3. Repetitive and longer exposure periods may also result in cumulative damage as demonstrated for vitamin E (Thiele *et al*, 1997a). In addition, ultraviolet radiation, a well-characterized oxidative stressor, is also present in areas of increased tropospheric O_3. Although we do not detect effects at lower O_3 levels in our model, the skin is exposed to several oxidative stressors simultaneously in real life, which will most likely show additive if not synergistic effects. Therefore, even lower levels of O_3 may be a relevant stressor in the presence of other oxidants. These cumulative effects and their biomedical implications must be the subject of further investigations.

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