Oxidative Stress and Antioxidants at Biosurfaces: Plants, Skin, and Respiratory Tract Surfaces

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Atmospheric pollutants represent an important source of oxidative and nitrosative stress to both terrestrial plants and to animals. The exposed biosurfaces of plants and animals are directly exposed to these pollutant stresses. Not surprisingly, living organisms have developed complex integrated extracellular and intracellular defense systems against stresses related to reactive oxygen and nitrogen species (ROS, RNS), including O_3 and NO_2. Plant and animal epithelial surfaces and respiratory tract surfaces contain antioxidants that would be expected to provide defense against environmental stress caused by ambient ROS and RNS, thus ameliorating their injurious effects on more delicate underlying cellular constituents. Parallelisms among these surfaces with regard to their antioxidant constituents and environmental oxidants are presented. The reactive substances at these biosurfaces not only represent an important protective system against oxidizing environments, but products of their reactions with ROS/RNS may also serve as biomarkers of environmental oxidative stress. Moreover, the reaction products may also induce injury to underlying cells or cause cell activation, resulting in production of proinflammatory substances including cytokines. In this review we discuss antioxidant defense systems against environmental toxins in plant cell wall/apoplastic fluids, dead keratinized cells/interstitial fluids of stratum corneum (the outermost skin layer), and mucus/respiratory tract lining fluids.— *Environ Health Perspect* 106(Suppl 5):1241–1251 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1241-1251cross/abstract.html*

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Atmospheric pollutants, largely arising as primary and secondary products of combustion, represent an important source of environmental oxidative stress to terrestrial plants, animals, and other organisms. As such, widespread attempts have been made to evaluate plant and mammalian responses to environmental oxidants, of which O_3, one of a number of potential environmental surface oxidants, has perhaps been the most studied. Many studies have focused on documenting such important end points as crop yield, forest decline, and human health (*1–4*). Recently, many pathobiologic studies have focused on the involvement of reactive oxidative species (ROS) (*5*) and the biologic antioxidant protective mechanisms induced by oxidative stress, especially with regard to antioxidant enzyme induction in the context of tolerance, e.g., ascorbate-specific peroxidases, catalase and superoxide dismutases in plants (*6–8*), and glutathione peroxidase, superoxide dismutases (SOD), and catalases in animals (*9–11*).

However, it is the outermost tissues of plants and animals that are initially directly exposed to gaseous environmental toxicants, including environmental oxidants. Their extracellular antioxidant defenses will be the first to encounter insult and may, in fact, be solely responsible for antioxidant defense in situations in which environmental oxidative stress fails to reach levels that penetrate these defenses to reach underlying cell membrane surfaces. Thus, in a sense, it is failure of this defense system that brings oxidants to the surface of underlying plasma membranes, causing injury to plant or animal cells.

The aim of this review is to highlight certain parallels between the antioxidant mechanisms responsible for protecting diverse environmentally exposed biosurfaces. Although there are numerous other environmental oxidants that contribute to surface oxidative stress (e.g., oxides of nitrogen, cigarette smoke, radiation), our focus will be on O_3 as a representative environmental oxidant and on the antioxidants with which it can be expected to react at plant, skin, and respiratory tract surfaces. Although in most circumstances the oxidant can be expected to be neutralized, products of reactions with constituents of the extracellular fluids, e.g., cytotoxic aldehydes produced by reactions of O_3 with extracellular lipids (*12–14*), may be largely responsible for cell or tissue injury by environmental toxins.

Structural Considerations

The similarities of the interfaces between the environment and plant and animal tissues are simplistically depicted in Figure 1. In plants, uptake of environmental toxins can be expected to occur mainly at the cell wall surface and, via the stomata, within the extracellular apoplastic fluids (*15–17*). The skin and the respiratory tract are the animal organs most directly exposed to oxidant air pollutants. Although numerous studies have documented the effects of oxidants on lungs, and have to some extent directed attention to the importance of the respiratory tract lining fluids (RTLFs) (*18–22*), only a few studies have described possible oxidative pollutant effects on cutaneous tissues (*23–26*).

RTLFs vary in different levels of the respiratory tract. The thickness of these layers has only been estimated, not rigorously

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Abbreviations used: BALF, bronchoalveolar lavage fluid; ELF, epithelial lining fluid (e.g., distal RTLFs); GSH, glutathione; MDA, malondialdyhyde; NLF, nasal lavage fluid; RNS, reactive nitrogen species; ROS, reactive oxygen species; RTECs, respiratory tract epithelial cells; RTLFs, $^{\bullet}NO_2$, nitrogen dioxide; $^{\bullet}OH$, hydroxyl radicals; $O_2^{\bullet-}$, superoxide anion radical; respiratory tract lining fluids; SOD, superoxide dismutase; UV, ultraviolet.

quantified. For example, as shown in Table 1, RTLF depth (both sol and gel layers) in the upper respiratory tract has been estimated to range from 1 to 10 μm, whereas in the distal bronchoalveolar regions, RTLF depth is only 0.2 to 0.5 μm (*18,27–29*). The composition of RTLFs also varies widely, with mucins present in upper RTLFs, whereas lower RTLFs contain surfactant proteins and lipids. Modeling studies have suggested that upper airway mucus does not flow evenly but preferentially concentrates along troughs or grooves (*30*). The quantitative description of antioxidants present at any given level of the respiratory tract is compromised by the complex gel–sol nature of the upper RTLFs and the probability that the mucin-containing gel layer is covered by a lipid layer that may be derived in part from the lower bronchoalveolar regions (*30–33*).

All three surfaces, plants, skin, and respiratory tract, represent important sites of environmental–biosystem interaction as sites of continuous reactive absorption of oxidant pollutants. Thus, in plants and animals, extracellular compartments can be considered an important sink for atmospheric oxidants, screening out to some extent the effects of oxidants on the underlying cells.

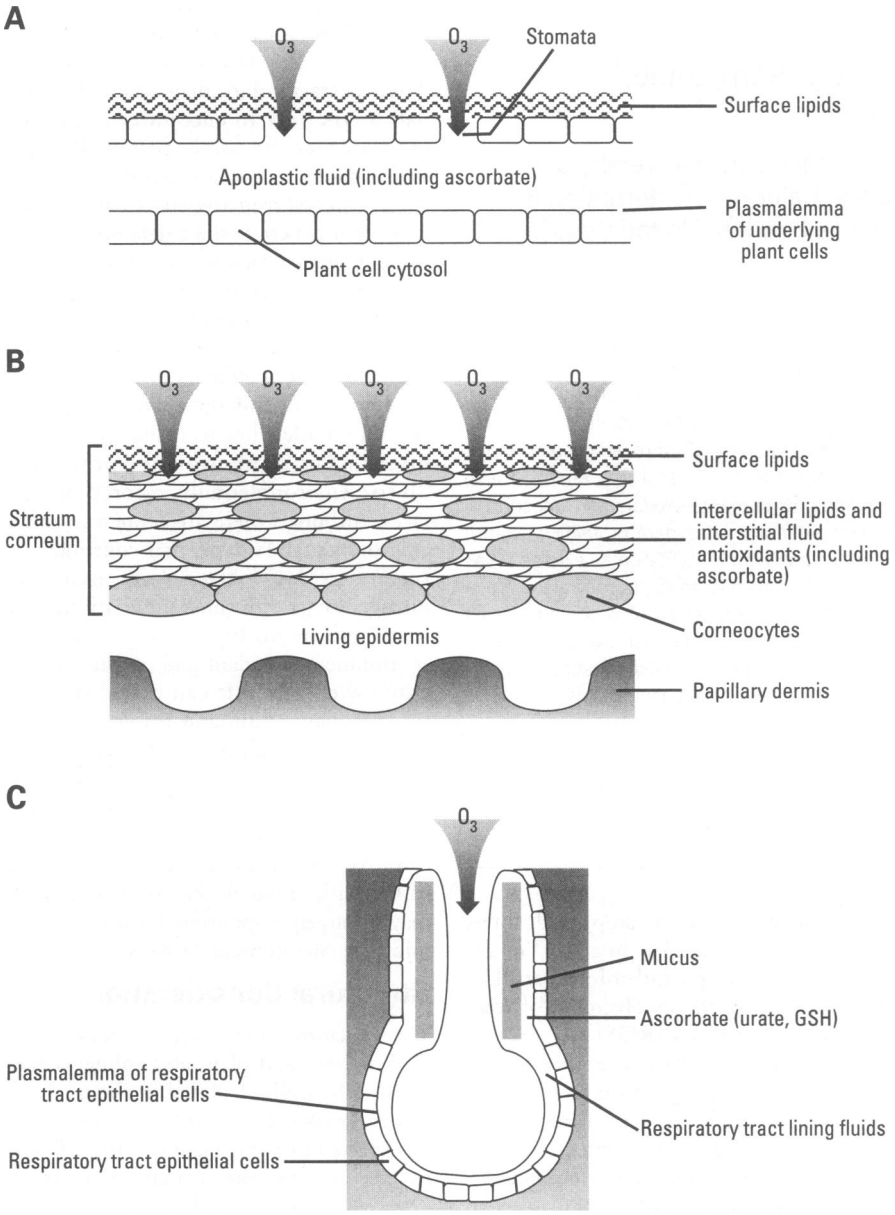

Figure 1. In (*A*) plants, environmental oxidants first react with components of the leaf surface, stomata, and extracellular apoplast before encountering the plasma membranes of the underlying cells. The apoplastic fluid contains substantial amounts of ascorbic acid. In animals, the inhaled environmental oxidant first encounters the (*B*) skin or the (*C*) RTLFs, and their contained antioxidants, including ascorbic acid.

Table 1. Human respiratory tract lining fluids.[a]

	Thickness, μm	Surface area, cm^2	Volume, ml	Turnover
Nasal	5–10	180	0.15	?>1000×/day
Airways	1–10	4500	3.5	?>20×/day
Alveoli	0.5–0.2	885,000	9	???

[a]Based largely on morphologic data (*18*).

Extracellular Antioxidants of Plants

Plants are no different from other aerobic organisms in that they are exposed to environmental toxins and have evolved to cope with oxidative stress (*5,34,35*) and in fact must cope with being O_2-producing organisms. Their antioxidant enzymes, such as ascorbate peroxidase (an H_2O_2-scavenging system more important in plants than in animals), catalases, and superoxide dismutases, are upregulated in response to several stresses, including oxidative air pollutants such as O_3 (*6–8, 36,37*). Other protective antioxidant mechanisms may include thioredoxin (*38*). Analogous to animal cells (*39*), almost any injury to plant cells can be expected to have the same consequence, i.e., the generation of oxidative stress. Thus, a general response to any injury would be expected to include an augmentation of antioxidant defense systems. However, with environmental oxidative stress, it is the extracellular constituents of the plant cell wall and apoplastic fluid that will first come into contact with oxidative challenge, and it is this compartment that can be expected to demonstrate parallels with animal skin and respiratory tract.

The biochemical and molecular mechanisms underlying O_3 toxicity in plants have begun to be unraveled in recent years (*5–7,16,17,34,40–52*), coincident with increased understanding of the complexity of antioxidant defense

systems employed by plants (*53*) and, parenthetically, the increasing appreciation of the nutrient value of plant antioxidants to animals (*39,54–56*). As depicted in Figure 1A, once O_3 enters the apoplast through stomata, it reacts quickly with biomolecules (including water) located there, and some of it may be converted into secondary ROS such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), H_2O_2, and singlet O_2 (*57–61*). Investigators have hypothesized, based on physical, anatomical, and kinetic considerations, that only under unusual conditions, or extremely high doses (much higher than usual air pollution levels), would O_3 permeate to the underlying plasma membranes (*62*). O_3 can react with some apoplastic biomolecules to generate toxic species (e.g., lipid peroxidation products) that could initiate damage to plasma membrane lipids and proteins. Such events could account for the decreased photosynthesis, electrolyte leakage, and accelerated senescence associated with exposure to toxic levels of O_3 (*47,48*).

It has been known for over three decades that ascorbate protects plants against O_3 injury (*40*), and, more recently, that mutants deficient in ascorbate metabolism pathways are sensitive to oxidants including O_3 (*52*). Ascorbate reaches up to 5-mM concentrations in apoplastic fluids (*17,41,43,45,63*). It has been calculated that this apoplastic ascorbate could provide an efficient protection against most ambient environmental O_3 concentrations (*43*). Of course, there are some important variables, also applicable to both animal cutaneous tissues and the respiratory tract, that must be taken into account. These include: *a*) the rate of ascorbate transport into the apoplastic fluid; *b*) the amount of O_3 reaching apoplastic fluid via the stomata (e.g., stomata, like animal airways, have variable resistances, and if this resistance is increased, less O_3 can be expected to penetrate into the apoplastic space); *c*) other reactions of ascorbate within the apoplastic fluid (e.g., utilization by the ascorbate peroxidase system and possible recycling of vitamin E in underlying plasma membranes); and *d*) possible regeneration of oxidized ascorbate by plasma membrane reductive mechanisms (*64*). With regard to the latter, it should be recognized that although ascorbate transport system(s) across the plamalemma of animal cells is a well-documented phenomenon (*65,66*), ascorbate transport system(s) across the plant plasmalemma are only beginning to be described (*67,68*) and, in fact, knowledge about the precise pathways and metabolic controls of ascorbate biosynthetic pathways is still incomplete (*52*). Importantly, dehydroascorbate (and perhaps more importantly monodehydroascorbate) reductase systems are more active in plant cells than in animal cells, although the specific reductase systems are multiple and vary in their adaptive responses to oxidative stress (*69,70*).

In addition to ascorbate, the apoplastic compartment contains a variety of other potential reactants for O_3 and its reactive products, including phenolic compounds and peroxidases. Analagous to the case for animals, it can be expected that much will be learned about plant antioxidant processes in the next decade via extensive experimental applications of transgenic plant technology (*71,72*). For example, if O_3 yields H_2O_2 in the aqueous phase of the apoplast, peroxidases found there or in the outer cell wall material, which is rich in peroxidases involved in lignin synthesis, could be protective via direct utilization of H_2O_2 (*8*). However, peroxidases may also represent a potential target for O_3 and can sometimes act as free radical generators, e.g., oxidation of thiols and NADH by plant peroxidases (*38*).

It is still an open question as to the mechanism by which O_3 damages plants, or indeed the respiratory tract. Again analogous to recently postulated mechanisms of respiratory tract O_3 injury (*12–14*), it is possible that hydrocarbons emitted by plants will react with O_3, yielding highly toxic peroxides, oxides, radicals, and other species. Two other possible parallels between plant and animal surfaces should be mentioned. All three surfaces (plant cell wall, skin stratum corneum, and respiratory tract mucus) contain lipids that could react with environmental oxidants as sacrificial targets, although O_3 reactions with surfactant proteins and lipids in the lower RTLFs could potentially have important pathophysiologic consequences (*12–14*). For example, plant outer cell wall surfaces contain waxes that react with O_3 (*73–75*).

A second parallel is that O_3 damage to skin and to lung initiates activation of inflammatory immune processes, in part via production of chemokines and cytokines by keratinocytes and respiratory tract epithelial cells (RTECs) in skin and respiratory tract, respectively. These inflammatory immune system activations include generation of ROS and RNS at animal surfaces, augmenting the oxidative stresses imposed by the environmental oxidant such as O_3. A somewhat similar process exists at the surface of plant cells, e.g., one effect of pathogens (including O_3) on plants is the triggering of the hypersensitivity response, which involves many components that are analogous to the NADPH-oxidase system of the animal phagocyte, e.g., an oxidative burst (*76*). This response normally defends against pathogens invading the plant (*77–82*) and may also be important in several signal transduction systems leading to modification of gene expression and may be involved in the induction of tolerance (*83–85*). However, overexuberance of this or related intracellular oxidative plant processes can also contribute to plant cell death (*85,86*).

Barrier Antioxidants of Skin

The cutaneous tissues of animals, along with the respiratory tract, represent the organs most directly exposed to environmental toxins such as O_3. In fact, O_3 may be among the most reactive chemicals to which the skin is routinely exposed in the environment. As schematically depicted in Figure 1B, the barrier compartment of the skin, the stratum corneum, is the site of the air/cutaneous tissue boundary. It comprises a unique system of structural, anucleate cells (corneocytes) embedded in a lipid-enriched intercellular matrix (*87*), forming stacks of bilayers that are rich in ceramides, cholesterol, and free fatty acids (88). This lipid–protein biphasic structure of the stratum corneum, having a thickness of only 10 to 20 μm, is believed to be the crucial determinant of the barrier function of the skin (*88–91*).

It is interesting to note that following acute injury to epidermal constituents *a*) local depletions of both enzymatic and nonenzymatic antioxidants may occur (*92*), *b*) that epidermal lipogenesis is stimulated, presumably to facilitate repair of barrier function (*93*), and *c*) important in considering skin responses to reactive environmental oxidants, outer noncellular layers of stratum corneum are continuously being shed. Thus, as these elements react with oxidants, they will desquamate, somewhat analogous to constituents of respiratory tract mucus in which reactants are removed from respiratory tract surfaces via ciliary clearance mechanisms.

In recent studies we exposed hairless mice to a single high dose of O_3 (10 ppm for 2 hr) and observed increased levels of malondialdehyde (MDA), a parameter of lipid peroxidation, in the whole skin,

although we were unable to detect depletion of antioxidants. However, topically applied vitamin E was substantially depleted after similar exposure to O_3 (*23*). Based on these considerations, we hypothesized that the oxidative effects of O_3 in skin occur mainly in the outer layers, and investigated the effects of a single dose of 10 ppm O_3 for 2 hr on three different layers of skin: upper epidermis, lower epidermis/papillary dermis, and dermis. Using this approach, Thiele et al. (*24*) demonstrated that this high O_3 level significantly depleted vitamins C and E and induced MDA formation in the upper epidermis, including the stratum corneum but not in underlying layers (*24*).

Using techniques based on sequential analysis of the layers of the stratum corneum by tape stripping (*87*), we were able to analyze the biologic effects of O_3 on the outer layers of skin, using much lower concentrations of O_3 (e.g., 1 ppm O_3). The sequential tape-stripping method removes approximately 20 ± 5 μg/cm^2 corneum per tape-strip procedure. As shown in Figure 2, stratum corneum vitamin E was depleted after *in vivo* exposure to O_3, and MDA formation was increased in a dose-dependent manner (*25*). Furthermore, repeated low-level O_3 exposures resulted in cumulative oxidative effects in the outermost stratum corneum layers. Compared to skin not exposed to O_3 (∝-tocopherol: 8.95 ± 1.3 pmol/mg wet weight, γ-tocopherol: 3.00 ± 0.3 pmol/mg), exposure to 1 ppm O_3 for 2 hr on each of 6 consecutive days resulted in vitamin E depletion (∝-tocopherol: 2.90 ± 0.6 pmol/mg, $p < 0.001$; γ-tocopherol: 0.5 ± 0.1 pmol/mg, $p < 0.001$), and MDA levels increased from 3.7 ± 0.3 to 4.5 ± 0.2 pmol/mg wet weight ($p < 0.01$) (*25*).

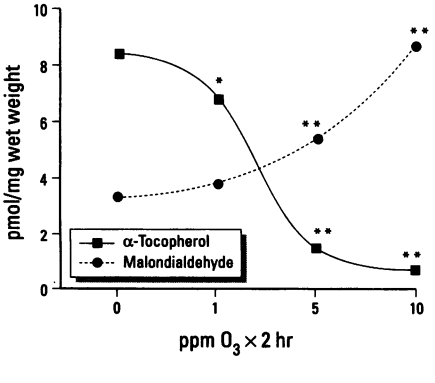

Figure 2. O_3 depletes vitamin E and induces MDA formation in the stratum corneum in a dose-dependent manner. ∝-Tocopherol (■) and malondialdehyde (●) determinations in pmol/mg wet weight extracts of stratum corneum of hairless mice exposed to the indicated dose of O_3 for 2 hr. *$p < 0.05$; **$p < 0.001$. $n = 4$ in each group (*22*).

O_3-induced lipid peroxidation in the stratum corneum may be harmful to skin in two ways. First, oxidation and degradation of stratum corneum lipids could affect the barrier function of the stratum corneum, as these lipids play an important role in barrier integrity (*87–91*). Perturbations of this outermost skin lipid and protein architecture have been suggested as important trigger factors for a number of dermatoses (e.g., psoriasis, atopic dermatitis, and irritant dermatitis) (*87*). The increase in MDA in the stratum corneum following O_3 exposure presumably involves oxidation of polyunsaturated fatty acids (PUFAs) such as arachidate and linolenate. This process may lead to significant changes in the lipid composition of the stratum corneum, which could conceivably affect epidermal function, perhaps to an extent limited by desquemation of dead cells.

Second, the increased formation of lipid oxidation products in upper skin layers could trigger injurious responses in adjacent skin layers. Reaction of O_3 with unsaturated lipids occurs via addition reactions (and breakdown to hydroperoxides, aldehydes, and H_2O_2) rather than via radical-mediated lipid peroxidation per se (*94*). Similarly, in the respiratory tract, 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 (*12–14,19,94*). Particular attention has been paid to the reaction of O_3 with lipids, although it is uncertain if this is the major damage caused by O_3 in biologic systems (*95–97*). 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 is believed to pass unreacted through a bilayer membrane, and none may pass through a cell (*19*). Secondary (or tertiary) O_3-induced lipid oxidation products, which have a lower reactivity and longer lifetime than O_3 itself, may transmit the effects of O_3 beyond the air/tissue interface. Because of their relative stability (at least when compared to many free radical species), lipid oxidation and peroxidation products, e.g., (hydroxy) aldehydes and cholesterol oxides, have the potential to damage cells at distant sites not directly exposed to O_3 (*98*).

Analogous with the respiratory tract surfaces, significant oxidative injury to the outermost layers of the skin can be expected to initiate localized inflammatory immune processes, resulting in the recruitment of phagocytes and their cell-specific, tightly regulated NADPH-oxidase systems for generating oxidants, thus amplifying the initial oxidative processes. In fact, the live keratinocytes underlying the corneocytes can themselves generate ROS, although quantitatively less than phagocytes, via their own sets of oxidative enzymes, including their plasma membrane NAD(P)H-oxidase, cytochrome P450, xanthine oxidase, monoamine oxidase, cyclooxygenase, and lipoxygenase systems (*99,100*). These systems can be turned on, to a degree, by cytokines, thereby adding further oxidative stresses at skin biosurfaces.

As in the case of O_3, there is evidence that ultraviolet (UV) light-induced skin damage, another environmental stress relevant to plants and to skin, is mediated in part by secondary products of oxidative processes (*101,102*). The formation of O_3 in the troposphere requires the presence of UV radiation, a known inducer of oxidative stress in skin (*101*). Because the O_3 exposures carried out in our studies were performed in the dark in a stainless steel chamber, and the animals were kept in the dark until killed, the observed oxidative stress effects were not influenced by UV irradiation. However, in urban pollution, the concomitant exposure to O_3 and UV radiation in photochemical smog potentially could cause synergistic oxidative stress effects in skin (e.g., UV can cleave H_2O_2 to two $^{\bullet}OH$ by homolytic fission, and many biomolecules can act as photosensitizers to generate oxidants after exposure to UV light). Although this synergy might also be important in plants and possibly in exposed skin, it seems irrelevant to the respiratory tract.

The inverse relationship observed in this study for vitamin E and MDA concentrations in stratum corneum following exposures to increasing O_3 concentrations (Figure 2), together with data that suggest that vitamin E may ameliorate UV radiation effects on keratinocytes (*103*) and on the skin (*104–106*), suggests a key role for this antioxidant in the prevention of oxidative damage in the skin. It may also be important in plants and in the respiratory tract (*107,108*). Furthermore, vitamin E acts as a penetration enhancer by intercalating within the lipid bilayer region of human stratum corneum, altering the characteristics of the membrane and affecting permeability (*109*). Thus, both vitamin E

content and lipid composition play major roles in permeability barrier homeostasis of the stratum corneum. It has recently been reported that ascorbate is also important in maintaining barrier lipid composition in reconstructed human epidermis (*110*). Interestingly, ∝-tocopherol appears to be more readily depleted by O_3 than γ-tocopherol, in accordance with findings in other tissues (*107*), which suggests a higher antioxidant activity for ∝-tocopherol (*25*). These findings may have implications for the pathophysiology of skin disorders, such as atopic dermatitis, that reportedly occur with increasing frequency in air-polluted areas (*111*). Finally, it should be noted that both ascorbate (*112,113*) and cysteine derivatives (*114*) including glutathione (*115*) appear to protect skin cells against UV radiation-induced oxidative stress.

Oxidative modification of biomolecules is believed to be a critically important step in the pathogenesis of tissue injury occurring secondary to exposure to environmental oxidants (*116*). The easy accessability of cutaneous tissues for bioassay purposes suggests that the stratum corneum could serve as a dosimeter for assessing environmental oxidative damage such as that caused by O_3. Such biomarkers have already been described for the case of O_3 reactions at respiratory tract surfaces (*117*). A review of biomarkers of oxidative damage to lipids, proteins, and DNA has recently been published (*118*).

Respiratory Tract Lining Fluids

The respiratory tract is covered by a thin fluid layer (RTLF) that forms an interface between the underlying RTECs and the external environment. The RTLFs thus constitute a first line of defense against inhaled toxic gases such as SO_2, O_3, $^{\bullet}NO_2$, and cigarette smoke. As schematically illustrated in Figure 3, constituents of these RTLFs can react with pollutants such as O_3 and may detoxify them to protect the underlying RTECs.

Studies with solutions of various antioxidants, and also with human blood plasma as a model extracellular fluid resembling RTLF, have demonstrated that O_3 reacts readily with ascorbate, urate, and thiols (*119–122*). Exposure of plasma to O_3 resulted in rapid depletion of its major water-soluble antioxidants, ascorbate and urate, at similar rates (*120,121*). As urate appears to be the most predominant low molecular mass antioxidant in proximal and nasal RTLFs (*123,124*) (see below), it has been hypothesized that urate may provide the most significant antioxidant defense against O_3.

There is much evidence that reactive gases such as inhaled O_3 and $^{\bullet}NO_2$ react with RTLF components and may never reach the underlying RTECs, at least in areas where they are covered by RTLFs (*12,19,61,125,126*). It follows that toxic actions of O_3 or $^{\bullet}NO_2$ toward RTECs are mediated, at least in part, by products of reaction of these inhaled toxins with constituents in RTLFs rather than by the direct interaction of these oxidant gases with RTECs. In the case of O_3, such toxins may include not only products from reactions with water, but also lipid hydroperoxides, cholesterol ozonization products, ozonides, aldehydes, and other oxidation products of antioxidants or proteins, as illustrated in Figure 3 (*12–14*).

As O_3 and $^{\bullet}NO_2$ are relatively insoluble, interactions of these inhaled gases with RTLFs are primarily governed by reactive absorption, i.e., the more oxidizable the substrate that is present in RTLFs, the more O_3 will be absorbed by the RTLFs (*122,126*). Therefore, inhaled O_3 or $^{\bullet}NO_2$ may be effectively removed by antioxidants present in the more abundant, proximal RTLFs to protect more distal RTLFs and RTECs. However, reaction of O_3 or $^{\bullet}NO_2$ with these antioxidants may generate secondary oxidants (e.g., $^1O_2$, $^{\bullet}OH$) (*127, 128*), which may injure RTECs in the upper respiratory tract, or result in cell activations designed to augment defense systems (e.g., increase vascular permeability, increase mucin secretion), or initiate inflammatory immune processes (*129*).

Because antioxidants in RTLFs may form the first line of defense against inhaled oxidants, it is important to understand the nature, chemistry, and fate of these antioxidants. Interest in the role of RTLF antioxidants in protecting against environmental oxidant pollutants has been stimulated by several observations. For example, uric acid, an important extracellular antioxidant, is thought to be secreted by the same upper RTECs that secrete mucin (*123,124*), and lower RTECs secrete α-tocopherol together

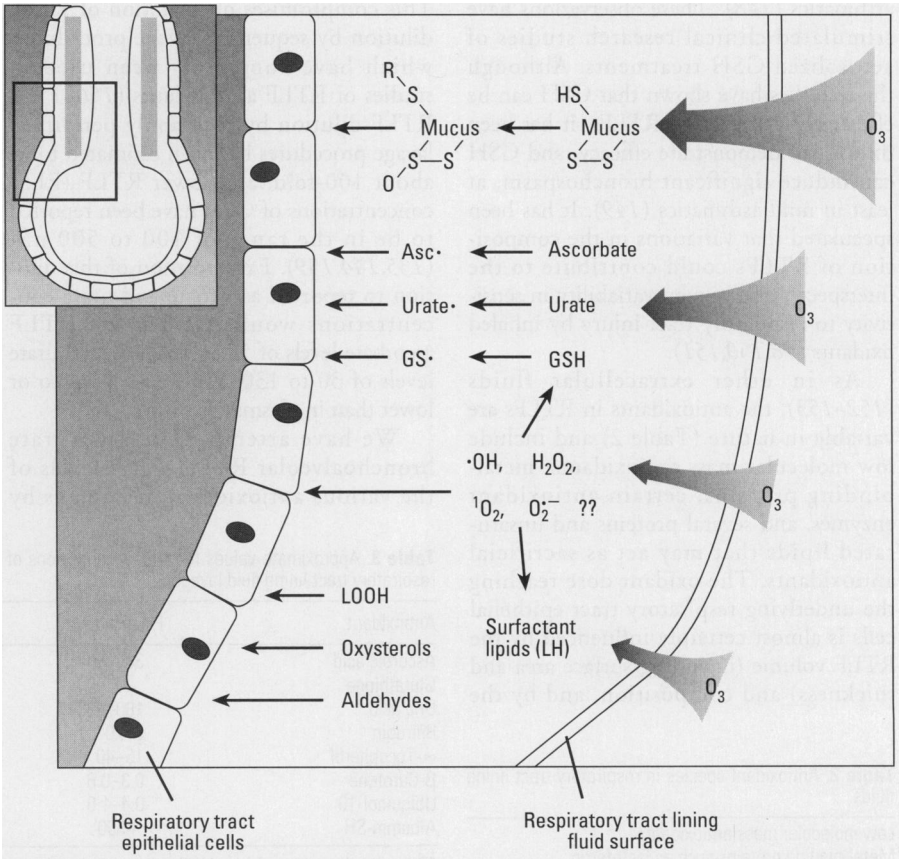

Figure 3. O_3 reactions with constituents of RTLFs. Note that as inspired O_3 is inhaled, its concentration is decreased secondary to its chemical reactions with the more proximal RTLFs. LH, lipid; LOOH, lipid hydroperoxide.

with surfactant (130), the latter being itself a target for oxidative injury (*131–134*). Bronchoalveolar RTLFs contain glutathione (GSH) in considerable excess of plasma levels and may contain more ascorbic acid than plasma (at least in some species) (*18,135*). Mucin itself (*136–138*), together with proteoglycans such as heparin sulfate and hyaluronic acid (*139,140*), has significant antioxidant properties, and in fact many toxicant irritants, including oxidants, can stimulate mucin secretion (*141*). In the case of mucins, both the abundant sugar moieties and the cysteine-rich domains would be expected to provide a rich antioxidant pool (*136*). It is regrettable that we know so little about the specific antioxidant functions of the respiratory tract mucins.

Interest in the antioxidant functions of the RTLFs has been further aroused by the observation that the GSH content of the lower RTLF appears to be subnormal in AIDS (*142*), idiopathic pulmonary fibrosis (*143*), cystic fibrosis (*144*), acute respiratory disease syndrome (*145*), and in lung allograft patients (*146*) but appears supranormal in cigarette smokers (*147*) and asthmatics (*148*). These observations have stimulated clinical research studies of aerosolized GSH treatments. Although these studies have shown that GSH can be effectively delivered to RTLFs, it has been difficult to demonstrate efficacy, and GSH can induce significant bronchospasm, at least in mild asthmatics (*149*). It has been speculated that variations in the composition of RTLFs could contribute to the interspecies and human variability in sensitivity to respiratory tract injury by inhaled oxidants (*18,150,151*).

As in other extracellular fluids (*152–153*), the antioxidants in RTLFs are variable in nature (Table 2) and include low molecular mass antioxidants, metal-binding proteins, certain antioxidant enzymes, and several proteins and unsaturated lipids that may act as sacrificial antioxidants. The oxidant dose reaching the underlying respiratory tract epithelial cells is almost certainly influenced by the RTLF volume (including surface area and thickness) and composition, and by the turnover rate of the various antioxidants present in the RTLFs (*18,149*).

Table 2. Antioxidant species in respiratory tract lining fluids.

Low molecular mass antioxidants
Metal-binding proteins such as lactoferrin
Antioxidant enzymes such as SOD and peroxidases
Sacrificial reactive proteins and unsaturated lipid

Several investigators have attempted to determine antioxidant concentrations in human RTLFs, which are commonly collected by nasal or bronchoalveolar lavage techniques. As summarized in Table 3, both nasal and bronchoalveolar RTLFs contain ascorbate, urate, and GSH as major water-soluble antioxidants. Moreover, it is readily apparent that nasal RTLFs contain relatively high levels of urate, whereas GSH appears to be the predominant low molecular mass antioxidant in distal RTLFs. Calculation of RTLF dilution by these lavage procedures has been attempted with the use of various dilution markers such as urea concentration. Measurements of urea are based on the hypothesis that extracellular urea concentrations are similar in all compartments, so that dilution of RTLF urea can be calculated by simultaneous measurement of plasma urea. This procedure is, however, hindered by the fact that instillation of urea-free saline causes a high concentration gradient, resulting in rapid diffusion of intracellular, interstitial, or intravascular urea into the lavage fluid. This compromises quantitation of RTLF dilution by sequential lavage procedures, which have commonly been used in studies of RTLF antioxidants (*156–158*). RTLF dilution by commonly performed lavage procedures has been estimated to be about 100-fold, and lower RTLF (ELF) concentrations of GSH have been reported to be in the range of 100 to 500 μM (*135,144,159*). Extrapolation of this dilution to reported ascorbate and urate concentrations would yield lower RTLF ascorbate levels of 50 to 180 μM and urate levels of 30 to 130 μM, i.e., similar to or lower than in plasma (Table 3).

We have attempted to quantitate bronchoalveolar RTLF (ELF) levels of the various antioxidants in humans by performing a single-cycle bronchoalveolar lavage procedure on twelve healthy nonsmoking volunteers, as described by Peterson et al. (*160*). This procedure can be performed rapidly (within 1 min) and therefore minimizes the diffusion of urea into the instilled saline so that RTLF dilution by this lavage procedure can be more accurately determined. The results indicated that RTLF levels of ascorbate are similar to those in plasma (40 ± 18 μM vs 63 ± 20 μM), whereas urate levels were somewhat lower (207 ± 157 μM vs 379 ± 167 μM) and GSH levels were much higher (109 ± 64 μM vs 1.0 ± 0.7 μM). Our results are in general agreement with most studies, and demonstrate that urate, rather than GSH, is the most abundant distal RTLF antioxidant (*161*).

Measurements of urea have also been used to estimate RTLF dilution by nasal lavage procedures (*158*). Collection of nasal RTLFs by instillation of 5 ml saline into one nostril for 10 sec has thus been estimated to result in approximately 10-fold dilution of nasal RTLFs (*158*). This implies that local antioxidant concentrations, as estimated from reported nasal lavage levels listed in Table 3, may range from 2 to 20 μM ascorbate, 20 to 250 μM urate, and 0 to 15 μM GSH. This suggests that antioxidant levels in nasal RTLFs are lower compared to bronchoalveolar RTLFs (especially true for GSH, and to a lesser extent ascorbate), but of course they contain considerable quantities of high molecular weight mucins, themselves potent antioxidants (*136*).

ELFs also contain several antioxidant enzymes and other proteins, as summarized in Table 4. Recently, it has become clear that lung cells secrete antioxidant enzymes such as extracellular glutathione peroxidase (*162*) or extracellular CuZn superoxide dismutase (SOD) (ecSOD or SOD-3) (*163*), which may contribute to the antioxidant defense in RTLFs. Recently, intracellular

Table 3. Approximate values for the concentrations of nonenzymatic antioxidants in human plasma compared to respiratory tract lining fluid lavages.

Antioxidant	Plasma, μM[a]	BALF, μM[b]	NLF, μM[c]
Ascorbic acid	30–150	0.5–1.8	0.1–1.5
Glutathione	<2	1.2–6.7	0.0–1.3
Uric acid	160–450	0.3–1.3	1.5–17.0
Bilirubin	5–20	–	–
∝-Tocopherol	15–40	0.02–0.05	–
β-Carotene	0.3–0.6	–	–
Ubiquinol-10	0.4–1.0	–	–
Albumin-SH	~500	~0.7	–

[a]Plasma values derived from Halliwell and Gutteridge (*152*) and Stocker and Frei (*153*). [b]BALF values largely derived from Hatch (*18*). [c]NLF data derived from Pacht et al. (*145*), Hatch et al. (*18*), Peden et al. (*124*), Housley et al. (*154*), and Testa et al. (*155*).

CuZnSOD (SOD-1) has been detected in bronchoalveolar lavage fluid (BALF) in addition to very small amounts of SOD-3 (*164*). Although some investigators have considered catalase (*165–167*) to be physiologically important in the RTLFs, there remains uncertainty whether it plays a significant role in the antioxidant defenses of RTLFs. It is possible that cytolysis, occurring as a natural process during cell turnover or occurring subsequent to lavage procedures themselves, contributes to the overall levels of extracellular enzymatic antioxidants demonstrated to be found in RTLFs. This holds true for GSH and ascorbate as well, as intracellular concentrations of these low molecular weight antioxidants are generally much higher than those found extracellularly. Because of its significant concentration in upper RTLFs, it is likely that secreted lactoferrin has a significant antioxidant function by binding iron and inhibiting iron-dependent free radical reactions.

Table 4. Antioxidant proteins known to be present in respiratory tract lining fluids.[a]

Metal-binding proteins	Other antioxidant proteins
Lactoferrin	Catalase
Ceruloplasmin	SOD-1 SOD-3
Transferrin	Glutathione reductase
Albumin	Glutathione peroxidase Ceruloplasmin (ferroxidase activity) Albumin (e.g., —SH activity)

[a]These proteins either bind metal ions in safe forms unable to catalyze damaging free radical reactions or possess other antioxidant properties (*152,165,166*).

Studies with blood plasma (as a model extracellular fluid) have indicated that exposure of plasma to O_3 or $^{\bullet}NO_2$ results in rapid depletion of ascorbate and urate, suggesting that these antioxidants provide the major defense system against O_3 (*95,168*). A recent study with a composite mixture of RTLF antioxidants has demonstrated that exposure to ambient O_3 concentrations results in depletion of primarily urate and ascorbate and to a lesser extent GSH. Moreover, these antioxidants prevent oxidative modification of proteins present in the mixture (*22*). It follows that urate and ascorbate provide a protective screen against inhaled O_3, and that GSH is relatively less effective (*122,126*).

A recent study with human volunteers has demonstrated that exposure to 2 ppm $^{\bullet}NO_2$ for 9 hr results in transient depletion of RTLF ascorbate and urate (*169*). RTLF GSH levels were not depleted but rather increased, again suggesting that ascorbate and urate are major reactants with inhaled oxidants and form the major first line of antioxidant defense in the respiratory tract.

What's Ahead?

Mechanistic understanding of plant and animal cell responses to environmental oxidant pollutants have blossomed over the past decade and continue to grow, receiving strong impetus from emerging academic disciplines of molecular environmental toxicology and driven by such resources as the National Institute of Environmental Health Sciences Environmental Genome Project. Existing plant (*15*) and animal models (*22,126,128*), and others to be generated, should provide important systems for studying the role of surface antioxidant species in modulating environmental effects on cell-signaling systems and gene expression pathways. The use of cell culture models for studying environmental oxidant effects, whether they are plant or animal cells, must recognize both the role of antioxidant species external to cellular surfaces in modifying potential end points *in vivo*, and also the role of extracellular antioxidants in maintaining intracellular levels of such important antioxidants as ascorbic acid and tocopherols. When extrapolations are made from data generated in such clean systems as *in vitro* primary, secondary, and immortalized cell culture systems exposed to environmental oxidants, allowances must be made for the contributions of extracellular agents at the environment–biosystem boundary under *in vivo* conditions. Finally, there is no doubt that studies of the effects of environmental oxidants on plant (*71,72*) and animal transgenics (*170*) in which components of antioxidant defense systems have been over- or underexpressed will contribute to the understanding of mechanisms of oxidative stress at plant and animal biosurfaces.

In conclusion, it is hoped this review will hopefully entice investigators to consider that surface biosystems could be playing an important role in their studies. Further scrutiny of similarites between the responses of plants, skin, and respiratory tract to insults such as O_3 can potentially contribute to the further understanding of mechanisms of oxidative environmental stress.

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