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Formation of Intracellular Free Radicals in Guinea Pig Airway Epithelium during *in Vitro* Exposure to Ozone

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***In vivo* exposure to ozone (O_3) has been shown to cause airway epithelial damage and lipid peroxidation. The oxidation of polyunsaturated fatty acids has been shown to produce hydrogen peroxide and aldehydes with reactive oxygen species (ROS) as intermediates. These products of ozonation may react with other bioorganic molecules and cause cellular damage. To assess the production of ROS, confluent primary cultures of guinea pig airway epithelial cells were grown on Costar membrane with a liquid–air interface and exposed to 0.2, 0.4, and 0.6 ppm O_3. The concentrations of intracellular ROS during the exposure were monitored using the fluorescent dye dihydrorhodamine-123. The intracellular concentration of ROS increased immediately upon the commencement of the O_3 exposure and persisted until the end of the exposure period (up to 1 hr). The concentration of ROS increased with increasing O_3 concentration. To determine the species of ROS produced during O_3 exposure, airway epithelial cells were perfused with dimethyl sulfoxide (DMSO), sodium formate (hydroxyl radical scavengers), NaN_3 (catalase inhibitor), or diethyl-dithio carbamate (DEDC, superoxide dismutase inhibitor) prior to and during the exposure period and the fluorescent intensity was monitored continuously. While both DMSO and sodium formate decreased the concentration of ROS, DEDC and NaN_3 had no effect. We concluded that hydroxyl radicals instead of H_2O_2 or superoxide anions were produced immediately following the commencement of O_3 exposure in guinea pig airway epithelial cells in an exposure concentration-dependent fashion.**

Ozone is a major health concern in the ambient air of urban communities throughout the United States. Controlled animal and human clinical studies as well as epidemiological investigations have documented respiratory impairment following ozone exposure (USEPA, 1989; Lippmann 1989). These include: reversible decrements in pulmonary function, increased permeability of epithelium, influx of inflammatory cells, stimulation of arachidonic acid release, and morphological pulmonary tissue damage. In laboratory animals, exposure to ozone can cause functional and structural alterations in respiratory tract (Wilson *et al.,* 1984; Nikula *et al.,* 1988; Tepper *et al.,* 1989). Morphologic responses of the extrapulmonary respiratory epithelium to ozone exposure *in vivo* include ciliary damage, necrosis, loss of ciliated cells, presence of intermediate cells, hyperplasia, and shifts in cell population densities (Nikula *et al.,* 1988; Wilson *et al.,* 1984; Harkema *et al.,* 1987). The location and intensity of these responses depend on the ozone concentration, animal species, duration of exposure, and time postexposure. Exposure of rats to concentrations between 0.5 and 0.9 ppm causes epithelial necrosis as early as 2 hr after initiation of ozone exposure, with the peak injury occurring by 24 hr (Stephens *et al.,* 1974a,b).

In vivo exposure to O_3 has been shown to cause airway epithelial damage and lipid peroxidation. The oxidation of polyunsaturated fatty acids has been shown to produce hydrogen peroxide and aldehydes with reactive oxygen species (ROS) as intermediates (Pryor and Church 1991; Pryor, 1992, 1994). These products of ozonation may react with other bio-organic molecules and cause cellular damage. To date, there are no data on the kinetics of ROS formation in airway epithelial cells exposed to ozone.

In order to better understand the mechanisms underlying the toxicity of ozone, it is necessary to control the variables which are inherent in *in vivo* exposures. Despite a number of organ culture studies, *in vitro* exposures of respiratory epithelium to oxidant gases and acidic particles have rarely been reported. Ozone-induced changes in the rate of glycoprotein secretion into the culture medium have been studied using *in vivo* exposures and subsequent culture of the preexposed tracheas (Last *et al.,* 1977). Similarly, *in vivo* exposures followed by culture of the preexposed tracheas have been used to examine the effects of ozone and sulfuric acid on ciliary beating frequency (Grose *et al.,* 1980).

An inclined rotating culture dish has been used to expose airway epithelial cells and PMN to ozone (Driscoll *et al.,* 1988; Valentine, 1985). However, this system is still not ideal in that it does not truly simulate the air–liquid interface which occurs in the airway lumen. The presence of a large volume of media in the inclined culture dish will further

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dilute substances secreted apically by these cells. Furthermore, since epithelial cells grown on plastic substrate are not nourished basolaterally, they do not organize themselves into a more columnar phenotype nor do they construct a continuous basement membrane or demonstrate distinct protein synthesis pattern profiles during differentiation (Whitcutt *et al.*, 1988; Adler *et al.*, 1987).

Recently, tracheal epithelial cells and alveolar macrophages were grown on rigid, collagen-impregnated filter supports and exposed to ozone with medium below the support but no medium on top of the cells (Cohen *et al.*, 1991). Using this system, exposure to ambient concentrations of ozone caused significant cytotoxicity to both cell types while no induction of stress proteins was observed. Similarly, Cheek *et al.* (1994) had shown that ozone exposure caused a dose-dependent increase in monolayer permeability, which resulted from damage to intercellular junctions and/or loss of epithelial integrity. In addition, Devlin *et al.* (1994) showed that although macrophages are much more sensitive to ozone than epithelial cells, they do not produce increased amounts of IL-6, IL-8, or fibronectin following ozone exposure. In contrast, epithelial cells (BEAS 2B) produce substantially more of all three proteins following ozone exposure, and both IL-6 and fibronectin are secreted vectorially.

We have recently developed a system capable of delivering liquid aerosols of uniform size at desired concentrations to target cells (Chen *et al.*, 1993). The system is capable of delivering acid particles over a wide size range, yet at constant particle mass concentrations. In this study, the system was modified to expose airway epithelium to the gaseous pollutant ozone at various concentrations. We monitored the kinetics of the production of ROS using a sensitive fluorometric assay. We found that hydroxyl radicals instead of H_2O_2 or superoxide anions were produced immediately following the commencement of O_3 exposure in guinea pig airway epithelial cells in an exposure concentration-dependent fashion.

METHODS

Airway epithelial cell culture. Male Hartley guinea pigs (virus free) weighing between 450 and 600 g were used in this study (Charles River Breeding Laboratories, Kingston, NY). Animals were anesthetized with ketamine hydrochloride (10 mg/100 g body wt, Aveco NADA 45-290) and their abdominal cavities were opened and phlebotomized via vena iliaca communis. The trachea and mainstem bronchi were removed, incised longitudinally, and incubated at 37°C in Hank's balanced salt solution containing 0.1% type XIV protease from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, MO) as described by Adler *et al.* (1990). The airway epithelium was removed by gentle scraping with a sterile No. 10 scalpel blade, disaggregated by repeated pipetting through a sterile pipette, and pelleted at 200*g* in a centrifuge at 4°C for 10 min. The epithelial cells were washed twice with Ham's F-12 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum before resuspension in supplemented Ham's F-12 medium supplemented with transferrin (5 μg/ml; GIBCO) insulin (5 μg/ml, bovine; GIBCO), cholera toxin (40 ng/ml; Sigma), epidermal growth factor (15 ng/ml; Collaborative Lab., Bedford, MA), hydrocortisone (360 ng/ml; Sigma), bovine pituitary extract (50 μg/ml; Collaborative Lab.), streptomycin (100 μg/ml; GIBCO), fungizone (0.5 μg/ml; GIBCO), and fetal calf serum (5% vol/vol; GIBCO).

The epithelial cells were plated on collagen-treated polycarbonate membranes (0.45-μm pore size; Costa, Cambridge, MA) mounted on a triangular cuvette as described below. Medium, above and below the cell culture, was replaced every 24 hr for the first 72 hr after plating. The medium above the cell culture was removed thereafter to maintain an air–liquid interface for the remaining culture period. Confluent cell layer was achieved in 10 to 14 days and was confirmed by examining the cell culture under an inverted microscope. Airway epithelial cells culture on the membrane embedded in plastic (3-μm section) revealed multiple layers of cells with cuboidal cells lying on the apical surface. Immunostaining demonstrated the presence of keratin (Dako kit), consistent with a population of differentiated epithelial cells.

Fluorescent dye loading. The production of ROS in airway epithelial cells during exposure to ozone was monitored by measuring dihydrorhodamine 123 (DHR) conversion as described by Royall and Ischiropoulos (1993). Twenty minutes before the commencement of the ozone exposure, epithelial cells grown on the membrane of the triangular cuvette were placed in another cuvette, kept in a thermostatic holder, and perfused at 0.6 ml/min with PBS buffer containing 5 μM DHR at 37°C. Rhodamine (RD, the conversion product of DHR) fluorescence was monitored continuously with excitation and emission wavelengths of 500 and 525 nm and excitation and emission slits of 1.8 and 4.5 nm, respectively, using a Spex Fluorolog spectrofluorometer (Edison, NJ). Immediately prior to ozone exposure, the excitation fluorescent spectrum of RD (at emission wavelength of 525 nm) was obtained for each cell preparation to ensure adequate dye loading.

Cell exposure/measurement system. An *in vitro* exposure system which monitors the intensity of intracellular fluorescent compounds of cells cultured on membrane during exposing to gaseous pollutants was developed for this study based on a system previously developed for acid aerosol exposure (Chen *et al.*, 1993). The schematic diagram of the system is shown in Fig. 1. The exposure/measurement system consists of a triangular cuvette fabricated from commercially available, disposable plastic cuvettes. A stainless steel template was used to make triangular cuvettes having reproducible dimensions. Polycarbonate membrane (the same material that was used in the Costa's Transwell) was glued on the surface of the triangular cuvette and allowed to coat with collagen and fibronectin at 37°C for 1.5 hr. Airway epithelial cells were grown on the membrane until confluent as described above. At the time of exposure, cell culture was placed inside a standard thermostated plastic cuvette (outer cuvette) and perfused from the basolateral side with buffer (described below) prior to ozone exposure using a peristaltic pump. An air–liquid interface was maintained at the apical side of the membrane/cell preparation (compartment B). A small tube was placed on one corner of the outer cuvette outside of the light path to remove fluid accidentally leaking into the compartment B. In almost all experiments, very little fluid was present in compartment B.

Airway epithelial cells were exposed to ozone at nominal concentrations of 0, 0.2, 0.4, and 0.6 ppm. The production of ROS was monitored as described above. After the exposure, cell viability was determined by measuring propidium iodide (PI) fluorescent labeling of nuclei in lethally damaged cells (Rojanasakul *et al.*, 1993). Briefly, cells on the membrane were stained with 1 μg/ml PI for 5 min and the fluorescence intensity (F_{smp}) was measured with an emission wavelength setting of 600 nm and an excitation wavelength of 490 nm, with excitation and emission slit widths of 4.5 and 9.0 nm, respectively. After a steady intensity was recorded, the maximum fluorescence intensity (F_{max}) was obtained by lysing the cells with 40 μM digitonin. The background fluorescence intensity (F_{bkg}) was obtained without the cell preparation in the cuvette under identical measurement conditions. The percentage of damaged cells was calculated according to the following equation:

$$\% \text{ damaged cells} = (F_{smp} - F_{bkg})/(F_{max} - F_{bkg}) * 100\%.$$

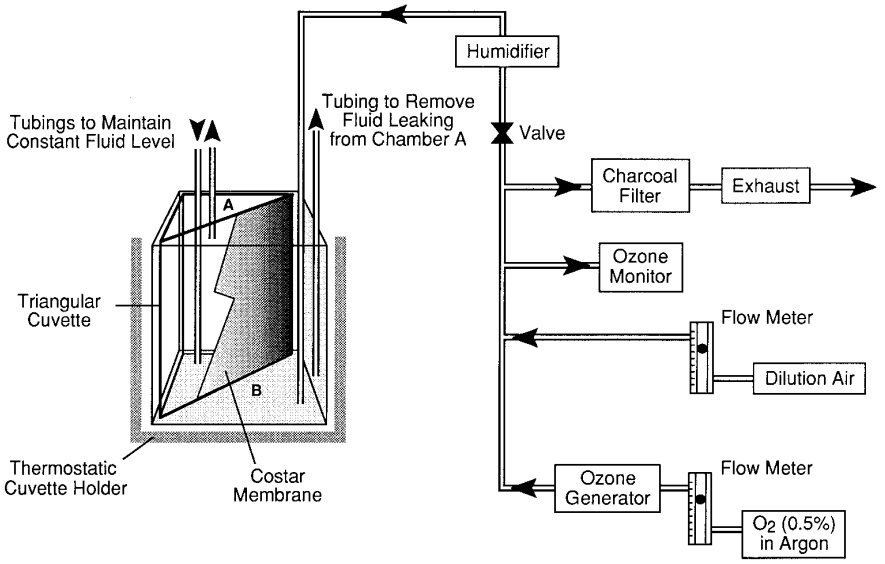

FIG. 1. The schematic diagram of the *in vitro* exposure system.

After measurement, cell preparations were stained with crystal violet to visualize the distribution of cells on the membrane. Data obtained from cell preparations that had uneven distribution of stained cells were discarded.

The ozone generation and exposure is shown schematically in Fig. 1. Ozone was generated by passing 0.5% O_2 (in argon) through an ultraviolet ozone generator (Sanders, Germany), diluted with filtered air (5% CO_2), warmed, and humidified before being delivered through Teflon tubing to compartment B of the outer cuvette. The exposure atmosphere was monitored continuously using an ultraviolet photometer (Monitor Labs. Model 8810). The monitor was calibrated daily using a certified UV transfer standard from a Monitor Labs Model 8550 calibrator. Since the flow rate (100 ml/min) of exposure atmosphere delivered to the triangular cuvette was too small to be sampled by the monitor, the exposure atmospheres were also collected in KI solution using an impinger and the absorbance of KI was used to calculate the actual delivered ozone concentrations.

Chemicals and buffer. Dihydrorhodamine 123 was purchased from Molecular Probes (Eugene, OR) and dissolved in dimethylformamide at a concentration of 20 mM as stock. The stock solution was purged with argon and kept at −20°C in desiccator. Phosphate-buffered saline with calcium (PBS) was selected as buffer for dye loading and cell perfusion during the measurement for DHR conversion. Dimethyl sulfoxide (DMSO) and sodium formate (both from Sigma) were chosen as hydroxyl radical scavengers to identify the possible role the hydroxyl radical may play in ozone-induced DHR conversion. Diethyldithiocarbamate (DEDC) and sodium azide (NaN_3, both from Sigma) were used as the inhibitors of superoxide dismutase (SOD) and catalase, respectively, in perfusion buffer to demonstrate the effects (if any) of superoxide anion and H_2O_2 on ozone-induced DHR conversion.

Data analysis. The average increase in fluorescent intensity of three cell preparations at each exposure concentration measured at 15 min after ozone was first introduced in the exposure system was used to perform statistical comparison between exposure groups. Statistical comparison of data sets between air and ozone exposure was performed using one-way analysis of variance. In all cases, statistical significance was accepted at $p < 0.05$.

RESULTS

Kinetics of ROS Formation in Airway Epithelial Cells during Exposure to Ozone

Figure 2 shows a representative fluorescent spectrum of RD (at emission wavelength of 525 nm). Since the fluorescent intensity of each cell preparation varied slightly due to variations in the dye loading as well as cellular density, the differences in fluorescent intensity between preexposure and those measured during exposure period were used. As shown in Fig. 3, the intracellular concentration of ROS increased immediately upon the commencement of the O_3 exposure as measured by increasing fluorescent intensity of RD. There was a short burst followed by a slower but sustained rise in fluorescent intensity until ozone was withdrawn from the exposure system, at which point the fluorescent intensity decreased rapidly. In addition, as shown in Fig. 4, the concentration of ROS increased with increasing O_3 concentration. No increase in fluorescent intensity was detected when the membrane without cells was exposed to the highest ozone concentration used in this study (0.66 ppm), indicating that ozone did not oxidize DHR to RD.

As shown in Fig. 5, there is a concentration-dependent

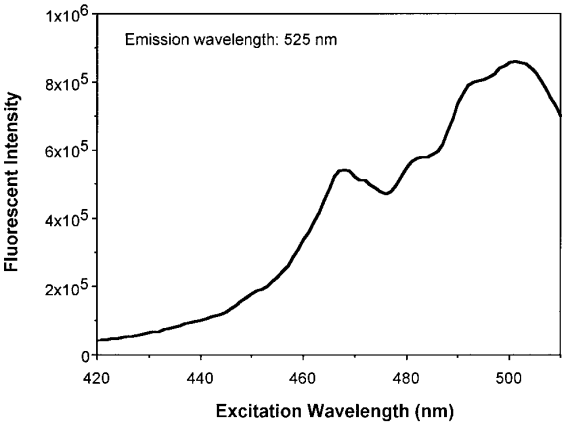

FIG. 2. The fluorescent spectrum of rhodamine 123 in guinea airway epithelial cells preloaded with dihydrorhodamine.

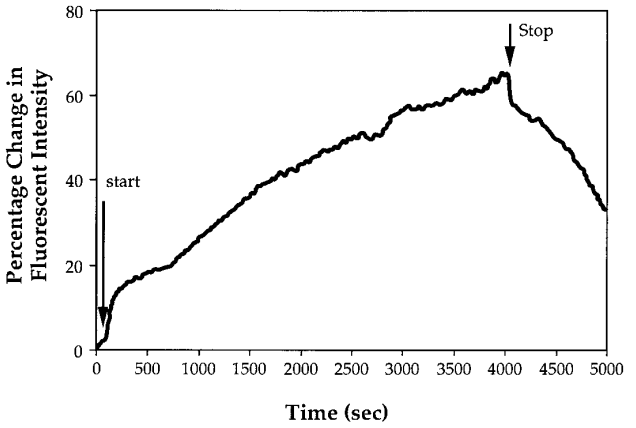

FIG. 3. Increase in fluorescent intensity of airway epithelial cells upon exposure to ozone. Airway epithelial cells were loaded with dihydrorhodamine for 20 min. Ozone at 0.44 ppm was introduced into the *in vitro* exposure system at the first arrow and stopped at the second arrow.

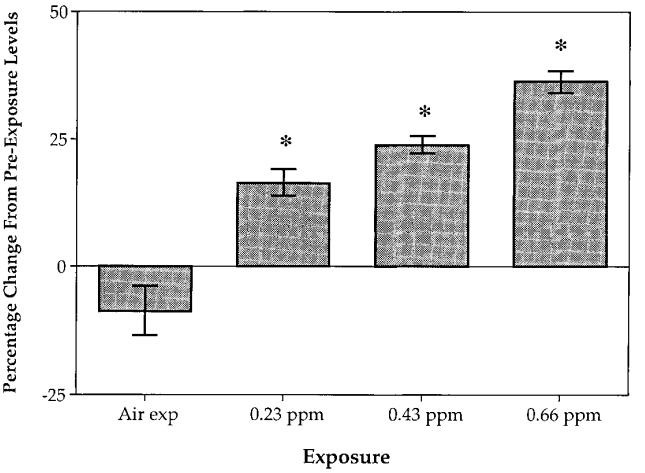

FIG. 5. Exposure concentration-dependent changes of ROS production in airway epithelial cells exposed to ozone.

increase in the ROS production in airway epithelial cells exposed to ozone. The slight decrease in fluorescent intensity in the air-exposed cells reflects either slight dye leaking or scavenging of ROS by endogenous scavengers. Figure 6 shows that the viability of the airway epithelial cells decreased with 0.43 and 0.66 ppm ozone, producing significant reductions in cell viability as compared to the control.

To determine the species of ROS produced during O_3 exposure, airway epithelial cells were perfused with DMSO (at a final concentration of 100 mM), sodium formate (100 mM), NaN_3 (1 mM), or DEDC (1 mM) prior to and during the exposure period and the fluorescent intensity was monitored continuously as described above. While both DMSO and sodium formate decreased the concentration of ROS, DEDC and NaN_3 had no effect. These data are shown in Fig. 7. Interestingly, as shown in Fig. 6, the reduction in ROS production by treatment of DMSO did not protect airway epithelial cells against ozone-produced cytotoxicity.

DISCUSSION

Ozone is a very reactive gas. Since airway epithelial cells are the primary cells that line the entire lung surface and are in direct contact with lung lining fluid, it is reasonable to assume that these cells are the primary target cells of ozone exposure. In humans and laboratory animals, exposure to ozone causes functional and morphological alterations in respiratory tract (Wilson *et al.*, 1984; Nikula *et al.*, 1988; Tepper *et al.*, 1989; Harkema *et al.*, 1987). The location and intensity of these responses depend on the ozone concentra-

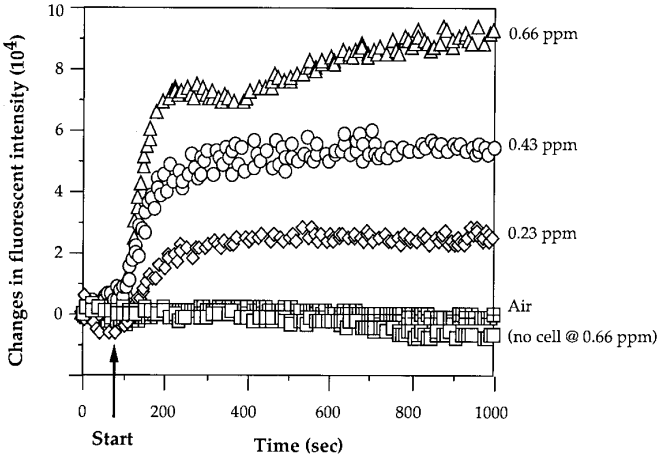

FIG. 4. Production of ROS increased in guinea pig airway epithelial cells with exposure to increasing concentrations of ozone. Without cells, no increase in fluorescent intensity was observed, indicating that neither membrane nor media is oxidized by ozone.

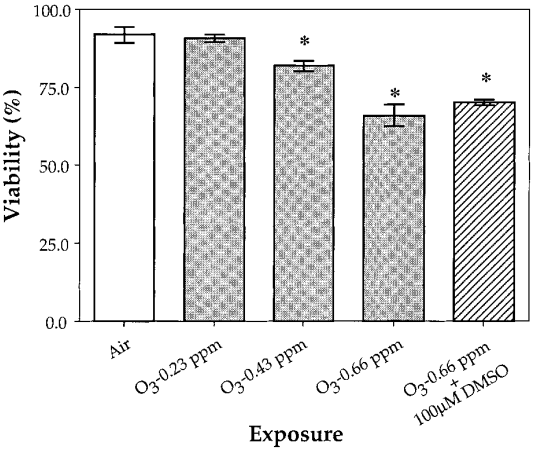

FIG. 6. Viability of airway epithelial cells exposed to increasing concentrations of ozone. Treatment of cells with 100 μM DMSO before and during the exposure did not attenuate the cytotoxicity of ozone.

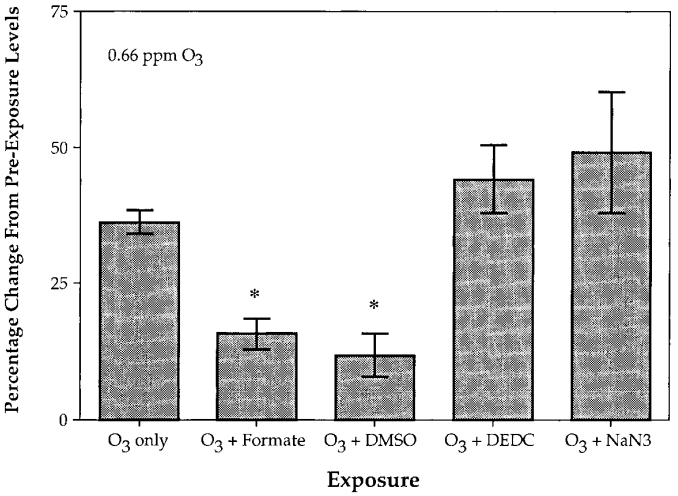

FIG. 7. Reduction in ROS production in airway epithelial cells exposed to ozone by treatment of cells with formate and DMSO before and during the exposure. DEDC and NaN_3 had no effect.

tion, animal species, duration of exposure, and time postexposure. Epithelial cells exposed to ozone can release a variety of mediators. For example, increased eicosanoid release from human airway epithelial cells exposed to these ozonolysis products has been observed (Leikauf *et al.,* 1993). These results imply that ozone can initiate biologic effects through an indirect mechanism that involves the reaction products of ozone and phospholipid. It is likely that epithelial cell activation and mediator release are the initial events in ozone-induced lung injury (Leikauf *et al.,* 1993).

Ozone reacts directly with biomolecules such as polyunsaturated fatty acids, some protein amino acid residues (cystein, histidine, methionine, and tryptophan), and some low-molecular-weight compounds (glutathione, urate, vitamins C and E, and free amino acids) in or on target cells or react with lung lining fluid (Mustafa *et al.,* 1990; Pryor and Church 1991; Pryor, 1992, 1994). Ozonolysis of an unsaturated fatty acid moiety in a membrane phospholipid results in scission of the carbon–carbon double bond, yielding an aldehyde and a hydroxyhydroperoxide (Santrock *et al.,* 1992) with ROS as intermediates (Pryor and Church 1991). These products of ozonation may react with other bio-organic molecules to give aldehydes and hydrogen peroxides. It is likely that these aldehydes and hydrogen peroxide are the species that relay ozone damage to more distant organs (Pryor and Church 1991; Pryor 1992, 1994).

Measurement of these intermediates has proved to be difficult. In most cases, only indirect evidence of ozonolysis products has been measured, at ozone concentrations higher than 1 ppm (Cueto *et al.,* 1992; Pryor *et al.,* 1992; Pryor, 1994). For example, Cueto *et al.* (1992) had shown the appearance of the aldehydes nonanal and heptanal in rat bronchial alveolar lavage fluid lipids after a 12-hr exposure to 1.3 ppm ozone. In the same laboratory, cholesterol ozonation products in whole lung tissue were also detected (Pryor *et al.,* 1992). Other markers, such as exhaled ethane and pentane, tissue measurements of diene-conjugates, and thiobarbituric acid-reactive substance, have been found to be relatively insensitive for use in inhalation experiments at ozone concentrations below 0.5 ppm (USEPA, 1989). In the current study, we have demonstrated direct evidence of intracellular free radical production in airway epithelial cells during exposure to ozone. Using DHR, the cytoplasmic ROS formation can be detected at ozone concentrations as low as 0.23 ppm and the formation of ROS increased with increasing ozone concentration. The nonfluorescent DHR is taken up by the epithelial cells and is converted during the ozone exposure into a green fluorescent compound, RD (Emmendorffer *et al.,* 1990; Royall and Ischiropoulos, 1993). This technique has been found to be more sensitive than other methods commonly used to investigate the production of ROS, such as the cytochrome c reduction assay, chemiluminescence, the nitroblue tetrazolium reduction assay, and assays based on the laser dye dichlorofluorescein (Emmendorffer *et al.,* 1990). The conversion rate of DHR due to basal cellular oxidizing reactions is relatively low (Royall and Ischiropoulos, 1993). When combined with leakage of the dye out of the cells, a net decline in the fluorescent signal was observed in air exposed cells (Fig. 3). Furthermore, since a very small quantity of fluid was leaking through the membrane, there is no direct conversion of DHR due to ozone exposure in a cell-free system (Fig. 3). Both DMSO and format reduced the fluorescent intensity during ozone exposure, and DEDC and NaN_3 (inhibitors of superoxide dismutase and catalase, respectively) had no effect. However, since we did not measure the activities of these enzymes directly, it is not possible to conclude that DHR is oxidized intracellularly by hydroxyl radicals, H_2O_2, or superoxide anions. Interestingly, reduction in ROS by DMSO was not accompanied by a reduction in cellular injury caused by ozone exposure. It is possible that DMSO did not completely interfere with the ozonolysis process or that the conversion of DHR may represent merely a side step in the ozonation cascade. Another possibility is that this assay measured the products of ozonation after cellular injury had occurred.

There are several limitations to this assay. Conversion of DHR can be mediated by a variety of reactions. Therefore, quantitative estimates of intracellular ROS are not possible. In addition, conversion of DHR cannot differentiate between oxidant-generating sources. For example, this assay will not differentiate ROS produced primarily by ozone from those produced by oxidative burst in activated macrophages. Nevertheless, this assay can be used qualitatively as a tool in monitoring overall ROS production in pollutant-exposed cells. In summary, the present study demonstrated that ROS, most likely hydroxyl radicals instead of H_2O_2 or superoxide anions, were produced immediately following the commencement of O_3 exposure in guinea pig airway epithelial cells in an exposure concentration-dependent fashion.

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