Ozone-induced Alterations in Glutathione in Lung Subcompartments of Rats and Monkeys

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The current studies were designed to test two hypotheses: (*1*) differences in steady-state reduced glutathione levels are responsible for subcompartment differences in susceptibility to acute ozone injury, and (*2*) elevation of reduced glutathione concentrations accounts for the tolerance to further injury produced by repeated ozone exposure. Glutathione was measured in well-defined subcompartments of the lung of both rats and monkeys to compare alterations occurring in both target (distal trachea and terminal bronchiole) and nontarget areas (lobar bronchus, major daughter, minor daughter bronchus, and parenchyma) of the lung in species that differ in sensitivity to ozone exposure (rat is less susceptible than monkey). Glutathione concentrations were decreased in trachea of rats exposed to 0.4 ppm ozone for 2 h and increased in lobar bronchus and distal bronchiole after 2 h exposure at 1 ppm. In monkey, glutathione levels in most subcompartments were not altered by either 0.4 or 1.0 ppm ozone exposure for 2 h. The exceptions were the major daughter subcompartment (200% of control at 0.4 ppm exposure) and the distal bronchiole (55% of control at 1 ppm exposure). Ninety day ozone exposures (6 h/day × 5 days/week) in rats produced an elevation in glutathione (164% of control value) only in distal bronchiole at the 1 ppm exposure level. In a similar manner, glutathione levels in the distal bronchiole of monkeys exposed for 90 days to 1 ppm O_3 were 165% of the corresponding control values. These results suggest the following: glutathione levels in target and nontarget areas of the lung and in susceptible versus less susceptible species are not the primary determinant in the differences observed in ozone toxicity; the response of lung subcompartments to short-term ozone exposure varied depending on airway subcompartment and species; increased glutathione levels may be one reason for adaptation of some airway epithelial cells from rats and monkeys exposed to O_3 for long periods; and use of well-defined segments of the lung provides a means of assessing changes in target areas of the lung without dilution from nontarget areas.

Ozone is a major component of photochemical smog and high levels of this pollutant are found in many urban areas worldwide. In cities such as Los Angeles, air concentrations of ozone frequently exceed the current National Ambient Air Quality Standard (NAAQS) of 0.12 ppm; in the summer, 0.2 to 0.3 ppm concentrations are not unusual (1). Although air quality control has achieved substantial improvement, the concentration of ozone is still far above the NAAQS in several U.S. cities (2). Levels of ozone in the 0.2 and 0.3 ppm range cause decrements in pulmonary function and produce inflammation characterized by elevation of polymorphonuclear neutrophils (PMN), macrophages, and proteins recovered from bronchoalveolar lavage of humans (3–5). These limited studies in the human are supported by extensive findings from animal experiments using both short-term and long-term exposure protocols. Ozone-induced injury is dependent upon the length of exposure and species. Short-term exposure to ozone in both nonhuman primates and laboratory rodents causes increased protein and inflammatory cells in bronchoalveolar lavage fluid (6), epithelial cell necrosis in trachea and bronchioles (7–9), and alveolar edema (10). Lungs from animals exposed to atmospheres with high levels of ozone show loss of normal epithelium, basal cell hyperplasia, and dysplasia in inferior nasal turbinates (11). Long-term exposure results in minimal epithelial injury and interstitial fibrosis (12), epithelial adaptation (13), and distal airway remodeling in rats (14).

Glutathione is a major intracellular thiol and has been found in high concentration in animals, plants, and microorganisms. A high ratio of reduced to oxidized glutathione is

(Received in original form June 10, 1995 and in revised form August 28, 1995)

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Abbreviations: diethylenetriamine pentaacetic acid, DTPA; nonprotein thiols, NPSH; phosphate-buffered saline, PBS; polymorphonuclear neutrophils, PMN.

critical in the maintenance of protein thiols which, in turn, are essential to the preservation of the structural and functional activity of many proteins (15). In addition, glutathione functions as a cosubstrate for two antioxidant enzymes, glutathione peroxidase and glutathione-s-transferase, which are thought to play an important role in cellular protection from ozone-induced injury (16). Glutathione reacts with aldehydes and fatty acid ozonides generated as byproducts in ozone-exposed tissue to form oxidized glutathione and nontoxic glutathione conjugates (17, 18). The importance of glutathione in modulating ozone-induced toxicity has been demonstrated both *in vitro* and *in vivo*. Depletion of glutathione in A549 cells markedly enhanced the sensitivity of these cells to ozone exposure (19). Likewise, treatment of mice with buthionine sulfoximine in the drinking water to deplete pulmonary nonprotein thiols (NPSH), markedly attenuated concentration-related increases in pulmonary NPSH after a 14 day ozone exposure and exacerbated the fibrotic response of the lung to these exposures (20). These studies suggest that glutathione plays an essential role in protecting the lung from the toxic effects of ozone.

The modulation of ozone-induced injury and development of tolerance to ozone is associated with increased antioxidant enzyme activity and glutathione levels in the lung (21, 22). However, a difficulty in interpreting the results of these experiments is that most of the data were derived from whole lung homogenates, yet the target areas for ozone in the lung are highly focal. More recently, we have assessed the changes in antioxidant enzymes in specific lung subcompartments and observed marked changes in some sites but not others (23). Thus, utilizing the whole lung to assess biochemical alterations associated with ozone exposure may lead to a considerable underestimation of the changes involved in both the initial cellular response to ozone exposure and the resistance that develops upon repeated exposure. The purpose of this study was to test two hypotheses: (*1*) that the focal nature of ozone injury is related to striking differences in the cellular glutathione content in target and nontarget areas of the lung, and (*2*) that the initial depletion and the subsequent high glutathione levels resulting from continued ozone exposure are associated with dramatic changes in target areas of the lung. Accordingly, this study reports changes in glutathione levels in well-defined lung regions prepared by microdissection in response to both short-term (2 h) and long-term (90 days) ozone exposure in two species which differ in susceptibility to ozone exposure (rat and rhesus monkey). This is the first study to examine changes in glutathione levels in well-defined regions of the lung in response to ozone exposure.

Materials and Methods

Experimental Animals

Male Fisher 344 rats (24 animals) were purchased from Simonson Laboratories (Gilroy, CA) at 4 to 5 wk of age. Animals were housed in high-efficiency particulate air (HEPA)-filtered cage racks with free access to food and tap water and were kept on a 12 h light/dark cycle. They were not used sooner than 7 days after receipt from the supplier. Rhesus monkeys (23 animals, age 3 yr and 2 mo to 3 yr and 10 mo, weighing 4.41 kg to 7.95 kg) were obtained from the California Regional Primate Research Center, University of California, Davis. They were housed in either outdoor caging or indoor controlled air environments prior to euthanasia.

Ozone Exposure

Ozone was generated by corona discharge using an OREC Model 03V5-Ozonator (Ozone Research and Equipment Corporation, Phoenix, AZ) with 100% oxygen. Ozone concentrations in each chamber were monitored by a multiplexed Dasibi Model 1003-AH (Dasibi Environmental Corporation, Glendale, CA) ultraviolet spectrophotometric analyzer. Animals were housed in modified Hazelton 2000 inhalation chambers and exposed to filtered air or ozone respectively. In one experiment, groups of four rats and rhesus monkeys (four to six animals per group) were exposed to filtered air, 0.4 or 1 ppm ozone respectively, for 2 h. In long-term exposure studies, rats (four animals per group) and monkeys were exposed to filtered air, or 1 ppm ozone respectively for 6 h per day, 5 days per week for 90 days.

Chemicals

Waymouth's medium MB752/1 containing 15 mM HEPES was obtained from Gibco Labs, Green Island, NY. Low gelling temperature agarose (Sea Plaque GTG) was purchased from FMC Bio Products, Rockland, ME. Glutathione (reduced form), *N*-2-hydroxyethylpiperazine-*N*-3-propanesulfonic acid, and *N*-ethylmaleimide, were purchased from Sigma Chemical Co., St. Louis, MO. Monobromobimane was obtained from Calbiochem, San Diego, CA. Diethylenetriaminepentaacetic acid (DTPA) was purchased from Aldrich Chemical Co., Milwaukee, WI. All chemicals were reagent grade or better.

Preparation of Tissue Specimens for Glutathione Determinations

The procedure for obtaining defined specimens of the lung by blunt dissection has been described in detail for rat and monkeys in a previous publication (24). Briefly, all rats were killed between 9 and 11 A.M. by an overdose of pentobarbital to reduce the effect of diurnal variation on glutathione levels. Monkeys were killed between 10:30 A.M. to 2:30 P.M. A small sample of liver tissue was removed to assure no large variations in glutathione concentrations as a result of differences in the time of death. No statistically significant differences were noted in hepatic glutathione levels in these tissues. The animals were exsanguinated and the trachea was exposed and cannulated. The lungs of rats were perfused with 20 ml phosphate-buffered saline (PBS) pH 7.4, via the pulmonary artery and 12 ml of low-temperature agarose (1% in Waymouth's medium) was injected via a tracheal cannula. Both rat and monkey lungs were immersed in Waymouth's medium for 30–60 min at 4°C. Beginning at the hilum of the left lobe of the rat and the right cranial lobe of the monkey, the tracheobronchial tree was removed by blunt dissection under a dissecting microscope (Wild M8; Heerbrugg, Switzerland). The following subcompartments were obtained from rat and monkey lung: distal trachea, lobar bronchus, the axial pathway of the largest (major daughter) branch, the pathways of the first and second of the largest daughter branches from the major axial pathway (minor daughters), the most distal three to four

TABLE 1
*Comparison of reduced glutathione levels in airway subcompartments of rat and rhesus monkey**

Lung Subcompartment	Rat	Monkey
Trachea	6.26 ± 1.45	7.09 ± 1.63
Lobar bronchus	5.38 ± 1.90	10.88 ± 2.96†
Major daughter airways	4.68 ± 0.76	5.00 ± 1.95
Minor daughter airways	6.06 ± 1.99	9.11 ± 2.65†
Distal bronchiole	8.25 ± 2.02	10.23 ± 1.78
Parenchyma	10.26 ± 3.21	12.10 ± 4.33

*Values are reported as nmoles/mg protein and the mean ± STD for eight rats and eight monkeys.
†Significant difference from the same airway subcompartment of rat at $P < 0.05$ (*t* test).

generations (distal bronchioles) of conducting airway, and lung parenchyma which was free of all of the other components. Preliminary studies have shown that glutathione levels do not vary over the time needed to complete the dissection (about 30 min in the rat and 40 min in the monkey) (25).

Glutathione Assays

Reduced glutathione was measured as the monobromobimane derivative by high-performance liquid chromatography (HPLC) (26). All samples were homogenized in a volume of 200 μl of 200 mM methanesulfonic acid containing 5 mM DTPA and 4 M sodium methanesulfonic acid and stored at −80°C for up to 1 wk. Defrosted samples were centrifuged at 9,000 *g* for 10 min. The pellets were used for protein determination and the supernatants were derivatized with monobromobimane at pH 8.0. The monobromobimane derivatives were separated by HPLC and the effluents were monitored with a fluorescence detector using excitation and emission wavelengths of 360 and 460 nm, respectively. Glutathione standards were run with each sample set and the response was linear over the range of sample values (20 pmoles to 2 nmoles).

Protein Assay

Precipitated protein was dissolved in 1 N NaOH and the amount of protein in the sample was determined by the Lowry method (27) with bovine serum albumin as a standard.

Statistical Methods

Results of glutathione assays are expressed as nmoles of glutathione per milligram protein and are presented as mean ± 1 standard deviation. Comparisons of glutathione concentrations between lung subcompartments and in lung subcompartments from air and ozone-exposed animals were done by a one-way analysis of variance (ANOVA). Post hoc tests were performed using Dunn's method, the Bonferroni method, or Student-Newman-Keuls's method to determine significant differences between airway levels. A P value < 0.05 was considered statistically significant.

Results

Glutathione levels were similar in comparable lung subcompartments of rats and monkeys except in the lobar bronchus and minor daughter airways. However, glutathione levels varied approximately twofold in different airways of the same species for both rats and monkeys (Table 1). In rats, glutathione concentrations varied from 4.68 ± 0.76 nmoles/mg protein in samples of major daughter airways to 10.26 ± 3.21 nmoles/mg protein in parenchyma. Glutathione levels in major daughter airways were significantly lower than parenchyma (Tables 1 and 2). Similarly, in rhesus monkeys, values for glutathione level in airway subcompartments of control monkeys varied from 5.0 ± 2.0 nmoles/mg protein in major daughter segments to 12.1 ± 4.3 nmoles/mg protein in parenchyma. Glutathione levels in the trachea and major daughter airways also were lower than in parenchyma. Glutathione levels in major daughter airways were lower than in the lobar bronchus, minor daughter and distal bronchiole.

Exposure of rats and monkeys to 0.4 ppm ozone for 2 h did not result in dramatic changes in airway glutathione (Figure 1). The only significant differences between filtered air control and ozone-exposed animals were observed in the trachea of the rat, where glutathione concentrations were depressed and in the major daughter airways of rhesus monkey where glutathione was elevated. In comparison, exposure for the same time period to high concentrations of ozone (1 ppm) caused a significant increase in glutathione levels in lobar bronchus, distal bronchioles, and whole lung of rats (Figure 1, top; $P < 0.05$), while in monkeys, the same exposures resulted in an apparent decrease in glutathione levels in the lobar bronchus, minor daughter and distal bronchiole segments of the lung. However, because of the variability in glutathione levels between animals, the only statistically significant differences were observed in distal bronchiole.

Long-term (90 day) ozone exposure at 1 ppm ozone appeared to result in slightly elevated glutathione levels in all airway subcompartments except the major daughter airway (Figure 2, top). These increases varied from 33% above control values to more than double the control level. Statistically significant differences in glutathione levels between filtered air and ozone-exposed animals were noted only in the distal bronchiole ($P < 0.05$). In monkey, long-term exposure to 1 ppm ozone significantly increased glutathione concentra-

TABLE 2
Comparison of reduced glutathione levels in different airway subcompartments of rats or rhesus monkeys

		Trachea	Lobar Bronchus	Major Daughter Airways	Minor Daughter Airways	Distal Bronchiole	Parenchyma
Rat	Parenchyma			*			
Monkey	Major Daughter airways		*		*	*	
	Parenchyma	*		*			

*Significant difference from the designated airway level within the same species at $P < 0.05$ (Dunn's method and Student-Newman-Keuls method).

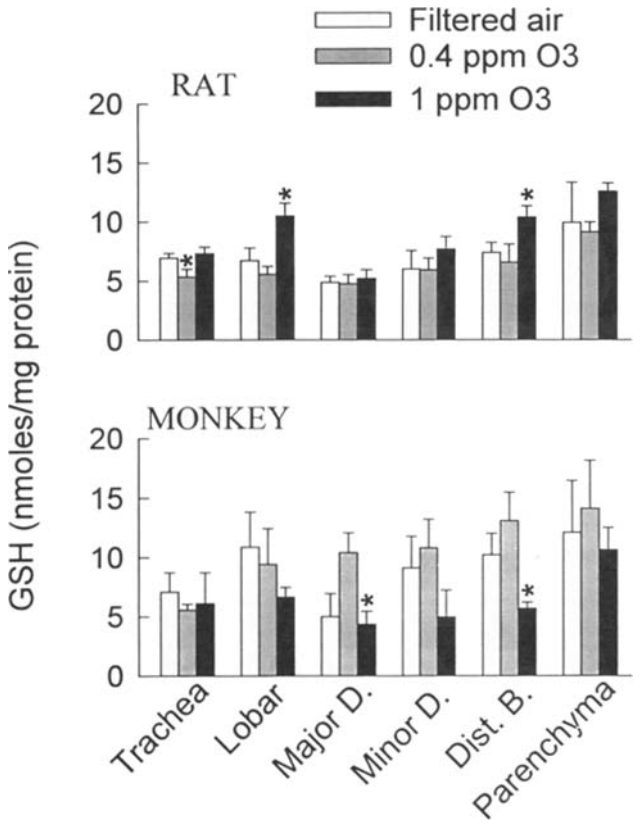

Figure 1. Glutathione concentrations (nmoles/mg tissue protein) in airway subcompartments after 2 h exposures to ozone at 0.4 or 1 ppm. Values are the mean ± STD for subcompartments obtained from 4 rats and 4–8 monkeys. * denotes a significant difference from control (filtered air) animals $P < 0.05$.

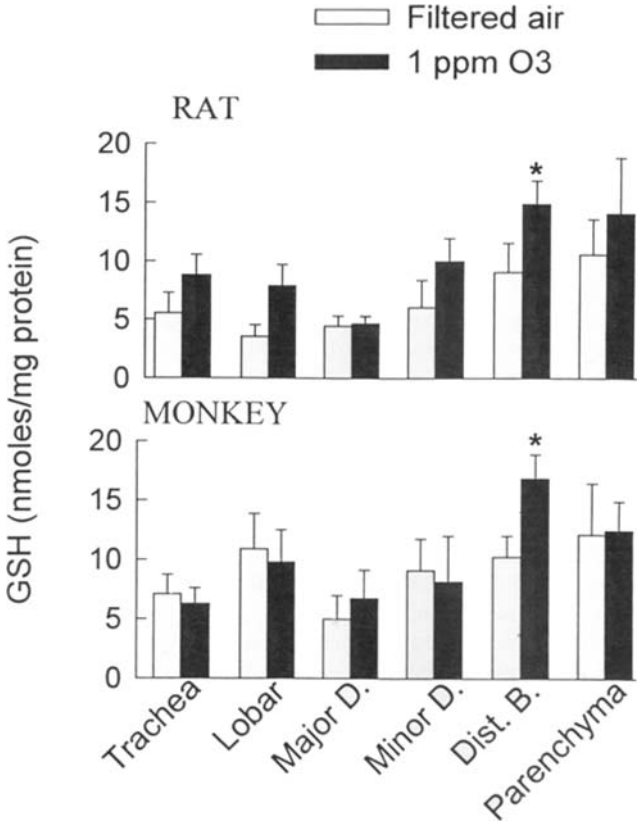

Figure 2. Glutathione concentrations (nmoles/mg tissue protein) in airway subcompartments after 90 day exposures to ozone at 1 ppm. Values are the mean ± STD for subcompartments obtained from 4–5 rats and 4–5 monkeys. * denotes a significant difference from control (filtered air) animals $P < 0.05$.

tions in the distal bronchiole (165% of control; Figure 2, bottom); in other compartments glutathione levels in ozone-exposed animals remained near levels observed in filtered air control animals.

Discussion

The goal of this study was to define the relationship between reduced glutathione pools and heterogeneity in the susceptibility of different regions of the respiratory system to oxidant stress produced by the inhalation of ozone. Different portions of the tracheobronchial airway tree vary in the degree of susceptibility to injury following short-term ozone exposure. Our study has demonstrated substantial differences in the glutathione content of different subcompartments of both rat and monkey lungs (Table 1). The finding that both the activity of protective enzymes (24) and glutathione levels in different airway subcompartments vary substantially underscores the need to measure biochemical effects of exposure to inhaled pollutants that produce focal injury, as does ozone, in well-defined areas of the lung. This view is further supported by data showing that activities of glutathione-metabolizing enzymes in homogenates of the whole lung do not necessarily reflect changes occurring in individual airway segments (23). Exposure of rats to ozone for 90 days resulted in a significant elevation of glutathione levels only in distal bronchioles (Figure 2 top). Likewise, a 2 h 0.4 ppm ozone exposure in the monkey caused a doubling in glutathione levels only in the major daughter airway segments (Figure 2).

Species and Airway Level Differences in Glutathione Levels: Lack of Correlation with Susceptibility to Ozone Injury

The differences in glutathione content of different airway levels do not correlate well with the relative sensitivity of different airways to ozone exposure. Target areas of the lung for ozone injury include distal trachea and centriacinus, containing the terminal bronchiole/alveolar duct junction (28, 29). The finding that levels of glutathione present in the distal bronchiolar airways of both rats and rhesus macaques were the same or higher than most of the airways studied, except the parenchyma supports the view that differences in glutathione levels within various airways are not the primary reason for regioselective injury by ozone. As shown in preliminary studies (30), local ozone dose may be important. In monkeys exposed to $^{18}O_3$, concentrations of oxidant were highest in the respiratory bronchiole. This finding is consistent with the data showing that the respiratory bronchiole is highly sensitive to ozone-induced cellular damage and that glutathione levels are altered significantly after both short-term, high dose, and long-term exposures (Figures 1 and 2). Likewise, differences in sensitivity of the rat and rhesus macaque to inhaled ozone (rhesus monkeys are more sensitive than rats) (29) do not correspond to significant differences

in glutathione levels in lung subcompartments. Thus, variations in cellular glutathione content or the activity of enzyme systems capable of protecting the cell from oxidant stress (which account for target site selective injury characteristic of ozone exposure) were not observed (24).

Alterations in Pulmonary Glutathione Levels after Ozone Exposure Are Not Associated with Marked Changes in Tripeptide Levels in Target Areas

Earlier work has shown that short-term exposures to relatively high levels of ozone (2 ppm) produce modest decreases in pulmonary nonprotein sulfhydryl content of rats (21). Two days after terminating the exposure, the nonprotein sulfhydryl content of the lungs increased to levels that are nearly twice control values. Since nonprotein sulfhydryl levels were expressed on a per lung basis, the initial drop in nonprotein sulfhydryls may be related to cell necrosis which occurs shortly after ozone exposure. The increases in glutathione level that occur later may be related to the proliferation of airway epithelium and/or the infiltration of macrophages which occurs after ozone exposure. In the present studies, glutathione concentrations were determined in well-defined lung subcompartments and were expressed on a per milligram protein basis, thus avoiding the problems associated with changes in glutathione content that are simply related to changes in the numbers of cells present in the airway subsegment. Glutathione concentrations were similar in comparable airways of rats and monkeys, but changes associated with ozone exposure were somewhat different. Trachea was the only subcompartment in which glutathione levels were altered in response to 0.4 ppm ozone in rats, whereas the same exposure in monkeys resulted in slight increases in most lung subcompartments with the exception of the major daughter segment where levels were nearly double control values. Likewise, after 1 ppm exposure, responses in rats and monkeys were different. Significant increases in glutathione were noted in the lobar bronchus and distal bronchiole after 1 ppm ozone exposure in rats, while a decrease was noted in the distal bronchiole of monkey. In both species, after both low and high dose exposures, changes in glutathione occurred selectively in target airways consistent with regional differences that have been observed in ozone exposure.

Long-term ozone exposure of rats to ambient concentrations (0.12 ppm for 90 days) produces virtually no change in the levels of glutathione in any of the airway subcompartments tested. At higher concentrations of ozone (1.0 ppm), glutathione levels were elevated in the distal bronchiole of both rats and monkeys. Again, the increase in glutathione levels in the distal bronchiole is consistent with data showing that this region of the airway is highly sensitive to short-term, high concentration of ozone exposure. Taken together, the findings that long-term, low dose and short-term, high dose ozone exposures produce significant alterations of glutathione in target lung areas suggest that the levels of the glutathione are probably important in protecting the epithelium from oxidative injury and may be a key factor in the tolerance which develops in distal airways to ozone exposure. However, glutathione levels do not appear to be the primary determinant of the site- and species-selective toxicity of ozone.

There are still several key issues which must be addressed to understand the importance of the changes demonstrated here, particularly after long-term ozone exposure. Although several studies have focused on the key processes controlling the intracellular concentrations of glutathione in lung cells (31, 32), none have addressed the possibility that long-term exposure to ozone or other pulmonary toxicants may alter key steps in the generation or degradation of this tripeptide. We are currently using the techniques described in these studies for providing defined segments of airway to examine the effects of both short- and long-term ozone exposures on the synthesis and regulation of intracellular glutathione levels.

Acknowledgments: This work was supported by NIEHS 00628, the Health Effects Institute, and a base grant to the California Regional Primate Research Center RR00169. UC Davis is a NIEHS Center in Environmental Health (ES 05707) and support for core facilities provided by the Center is gratefully acknowledged.

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