HEALTH EFFECTS INSTITUTE

Morphometric Analysis of Alveolar Responses of F344 Rats to Subchronic Inhalation of Nitric Oxide

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Includes the Commentary of the Institute's Health Review Committee

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HEI Statement

Synopsis of Research Report Number 88

Alveolar Changes in Rat Lungs After Long-Term Exposure to Nitric Oxide

BACKGROUND

Nitric oxide (NO) in the environment is produced by the combustion of fossil fuels and tobacco. At high concentrations, NO is considered an environmental pollutant and, when inhaled, may be converted to several potentially toxic nitrogen-containing compounds, which can have complex diverse effects in people. However, NO is also formed in the body and, at low concentrations, mediates some important physiologic functions. It also has been used therapeutically to treat several pulmonary conditions, including adult respiratory distress syndrome and pulmonary hypertension.

No National Ambient Air Quality Standard for NO has been established, although NO in the atmosphere is readily converted to nitrogen dioxide (NO_2), and the National Ambient Air Quality Standard for NO_2 may provide some control of NO levels. Ambient concentrations of NO generally range from 0.01 to 0.18 parts per million (ppm); little is known about health effects or the range of exposures that may be toxic. Dr. Mercer previously reported that rats exposed to 0.5 ppm NO developed holes in the alveolar septa, the walls that separate individual tiny air sacs (alveoli) in the lung. He also reported that the number of holes was significantly greater in animals exposed to NO than in animals exposed to NO_2. The Health Effects Institute supported the follow-on study described in this report to learn more about the toxicity of NO so as to compare it with two other important oxidants, ozone and NO_2.

APPROACH

Dr. Mercer exposed three groups of rats continuously for six weeks to 2 or 6 ppm NO or to filtered air. The NO was free of ozone and NO_2. At the end of the exposure period he used an electron microscope to measure the number of holes in the alveolar septa and to observe other structural changes, such as in the surface area and the number and type of other abnormalities in the alveolar septa. The investigator also collected samples of fluid from the large and small airways and analyzed them for markers that would indicate inflammation and cell damage. He compared these results with those obtained previously in experiments using ozone and NO_2.

RESULTS AND INTERPRETATION

Dr. Mercer found no increase in the number of holes in the alveolar septa and no change in the thickness of individual alveolar wall compartments in animals exposed to 2 or 6 ppm NO compared with control animals exposed to air. These results differ from those of an earlier study, in which he and his colleagues used a lower concentration of NO (0.5 ppm). The discrepancy between findings of increased holes in the alveolar septa in Dr. Mercer's previous study and no increase in the present study is an unaddressed issue and possible explanations should be explored. Analyses of the lung fluid showed no increase in chemical or cellular indicators of inflammation in animals exposed to NO. This study is one of the first to explore the toxicity of NO on lung structure and establishes a basis for future studies of the health effects of this air pollutant and therapeutic agent.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1995 through 1997. This study was conducted by Robert R. Mercer at the Department of Medicine, Duke University, Durham, NC. The following Research Report contains both the detailed Investigator's Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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INVESTIGATOR'S REPORT

Morphometric Analysis of Alveolar Responses of F344 Rats to Subchronic Inhalation of Nitric Oxide

Robert R. Mercer

ABSTRACT

Nitric oxide (NO)*, the principal airborne pollutant generated from combustion processes such as gas stoves, tobacco smoke, and burning of fossil fuels, is being tested as a therapeutic agent in clinical trials. A prior morphometric study of rats exposed for 9 weeks to 0.5 parts per million (ppm) NO demonstrated focal degeneration of the alveolar interstitium and increased numbers of fenestrated alveolar septa (Mercer et al. 1995). The limited size and distribution of defects in this NO exposure did not alter alveolar surface area or other morphometric indicators of lung function, but were of interest as the responses to inhaled NO appeared to differ from those produced by other oxidants such as ozone (O_3) and nitrogen dioxide (NO_2).

Nitric oxide exposures at the same concentration and duration as prior morphometric studies of O_3 and NO_2 were necessary in order to make a comparison. This was the purpose of the current study in which F344 rats were exposed for 6 weeks to air, 2 ppm NO, or 6 ppm NO.

Following exposure, the lungs of NO- and air-exposed rats were preserved and prepared for electron microscopy. The lungs of replicate groups were lavaged and analyzed for protein content and antioxidants. Ultrastructural alterations due to exposure were determined by quantitative morphometric analyses and serial-section counts of the number of alveolar fenestrae. In contrast to the prior study of NO, there was no significant difference in the number of alveolar fenestrae/lung between control and NO-exposed groups. Morphometric analysis of the 6 ppm NO-exposure group demonstrated a significant increase from controls in the percentage of epithelial basement membrane covered by type II epithelial cells and a significant increase in the number of type II epithelial cells and airspace macrophages. At 2 ppm, only the percentage of epithelial basement membrane covered by type II epithelial cells was significant. No significant differences were found in lavage protein or in lavage ascorbic acid or glutathione content between clean-air controls and NO-exposed groups. Overall, the proinflammatory responses by type II epithelial cells and airspace macrophages following inhaled NO were comparable to those of O_3 and NO_2. These results, derived from experiments using significantly higher concentrations than in the prior study, demonstrate that inhaled NO produces a pattern of injury similar to that of other oxidants.

INTRODUCTION

PHYSIOLOGY AND PATHOPHYSIOLOGY OF NITRIC OXIDE

Unlike other inhaled pollutants, NO is a normal, endogenous, physiologic agent of the body, which mediates vasodilation, neurotransmission, phagocyte killing of bacteria, and inhibition of platelet aggregation (Beckman 1991). Nitric oxide is produced by a variety of pulmonary cells such as endothelial cells, type II epithelial cells, macrophages, and neutrophils (Kobzik et al. 1993; Robbins et al. 1994). As the understanding of NO function has expanded, a variety of beneficial anti-inflammatory effects and toxic proinflammatory responses has been determined.

Anti-inflammatory responses of NO include inhibition of platelet aggregation (Alheid et al. 1987); inhibition of cytokine-induced expression of adhesion molecules

* A list of abbreviations appears at the end of the Investigator's Report.

This Investigator's Report is one part of Health Effects Institute Research Report Number 88, which also includes a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigator's Report may be addressed to Dr. Robert R. Mercer, Pathology and Physiology Branch, National Institute of Occupational Safety and Health, Morgantown, WV 26505.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private-party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

(DeCaterina et al. 1995); inhibition of leukocyte adhesion (Kubes et al. 1991); and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which prevents neutrophil respiratory burst (Forslund et al. 1995). The toxic, proinflammatory action of NO is thought to be due to the formation of the potent pulmonary toxicant peroxynitrite (Beckman 1991). The role of this toxicant in lung disorders has been demonstrated by studies that show widespread distribution of peroxynitrite production in patients with acute lung injury and in animal models (Haddad et al. 1994).

Superoxide reacts with NO at nearly diffusion-limited rates to form the toxicant peroxynitrite (Beckman and Koppenol 1996). Upon diffusion of NO into the tissue spaces of the lungs, the production of peroxynitrite may occur in any lung space where superoxide is present. The liquid lining layer and the intracellular spaces of the lungs have significant concentrations of antioxidants, which efficiently scavenge superoxide (Slade et al. 1993; Cross et al. 1994) although the extracellular spaces are less well protected and are likely to be sites of significant peroxynitrite production (Beckman 1991).

NITRIC OXIDE EXPOSURE EVALUATION

In addition to its role as an endogenously produced physiologic mediator, NO is also a common airborne pollutant. Of the oxides of nitrogen (NO_x), NO is the primary oxide formed from combustion processes and environmental exposure poses a significant health risk to humans. Examples are ambient outdoor exposure (U.S. Environmental Protection Agency 1991), indoor exposure in homes due to outdoor air as well as endogenous gas sources (National Research Council 1986), and exposure to mainstream and sidestream tobacco smoke (National Research Council 1986). In the atmosphere, NO reacts with O_3 to form NO_2, which may photolyze to reform NO and O_3. Because of these interconversions, the ambient concentration of NO and NO_2 is a function of sunlight intensity and duration, air mixing, and the residence time of the gas. In exposure assessments, NO is found to be present at concentrations equal to or greater than those for NO_2 in both indoor and outdoor environments (National Research Council 1986). For instance, mean outdoor concentrations in the United States range from 0.01 to 0.12 ppm for NO and from 0.03 to 0.06 ppm for NO_2 (National Research Council 1986). Although indoor concentrations of NO_x are typically comparable to those in the outdoor environment, indoor concentrations where sidestream smoke is present are often higher with average NO concentrations ranging from 0.01 to 0.18 ppm and NO_2 concentrations from 0.02 to 0.08 ppm (National Research Council 1986). Daily spikes due to gas cooking and heating in homes and heavy traffic in the outdoor environment result in transient exposure to concentrations many times greater than these averages (Wade et al. 1975; National Research Council 1986).

In addition to this low-level exposure, there are several significant sources of human exposure to high concentrations of NO. Exposure to NO occurs from tobacco smoke where the concentration may be as high as 1,000 ppm (U.S. Environmental Protection Agency 1991; National Research Council 1986). High-concentration exposure also occurs in clinical settings where NO is used as a therapeutic agent, such as in the treatment of pulmonary hypertension (Pepke-Zaba et al. 1991; Kinsella et al. 1992). Concentrations on the order of 6 ppm are being tested for efficacy in infants with pulmonary hypertension (Pepke-Zaba et al. 1991; Roberts et al. 1992). It is apparent that human exposure to NO and NO_2 results from a variety of indoor and outdoor sources as well as from therapeutic intervention, which can be virtually continuous for long periods of time.

DOSIMETRY OF INHALED NITRIC OXIDE

Assessing pulmonary toxicity that occurs as a result of exposure to NO is hampered by the potential for experimental NO-containing atmospheres to be contaminated by NO_2, which is a recognized potent pulmonary toxicant. Under common environmental conditions, NO is oxidized to NO_2 with a concentration-dependent half-life, which varies from a few minutes at 1,000 ppm to 2.5 hours at 20 ppm (Borland and Higenbottam 1985). The conversion of NO to NO_2 in the atmosphere is also significantly enhanced in the presence of O_3 (U.S. Environmental Protection Agency 1991). At normal concentrations of O_3 and oxygen, O_3-mediated conversion is the predominant mechanism with a typical half-life of 20 minutes (National Research Council 1986). Because the conversion from NO to NO_2 in the atmosphere is rapid, the usual air-exchange rate in pollutant-exposure chambers is insufficient to prevent conversion; most studies alleged to be to either NO or NO_2 exposures actually contain unknown combinations of the two gases. To administer relatively pure exposures to NO or NO_2, one needs to use a pure source of NO_x with a relatively high exchange rate and/or selective removal in the exposure chamber.

We note that the conversion of NO to NO_2 once inhaled into the lung is not significant. The 20-minute half-life for conversion cited above is significantly longer than the time required for the washout of a single tidal breath (Borland and Higenbottam 1985). Isotopic studies have demonstrated that inhaled NO is efficiently transported to the

lungs and combined with blood in the pulmonary circulation (Yoshida et al. 1980). Most inhaled NO crosses the alveolar capillary barrier and binds with hemoglobin in red blood cells with relatively minor consumption by other lung tissue reactions. For instance, Yoshida and colleagues (1981) found that less than 10% of inhaled NO was removed in a bloodless rabbit lung. On the other hand, absorption in humans and rats with normal circulations is over 85% complete (Wagner 1970; Yoshida et al. 1980). These studies as well as others that have used NO to measure the diffusion capacity of the lungs (Meyer and Piiper 1989) demonstrate that NO passes rapidly from the airspaces to the circulatory system.

TOXICITY OF INHALED NITRIC OXIDE

The few studies that have used appropriate exposure techniques to study chronic low-level exposure to pure NO have shown substantial pulmonary effects (Azoulay et al. 1977; Holt et al. 1979). Azoulay and colleagues (1977) found that rats exposed continuously to NO (2 ppm) for 6 weeks had significantly enlarged airspaces and destroyed alveolar septa. This finding is particularly important as these two morphological features are central aspects of the anatomical definition of human emphysema (National Institutes of Health 1985). At higher (10 ppm) and longer exposures (up to 30 weeks), Holt and colleagues (1979) found grossly emphysematous lungs in NO-exposed mice; comparable exposures to NO_2 demonstrated only airspace enlargement.

COMPARISON WITH NITROGEN DIOXIDE TOXICITY

In part, the absence of studies on NO toxicity reflects the focus on NO_2, which has a greater reactivity and readily produces lung inflammation and injury in acute exposures. Nitrogen dioxide principally reacts with cell membranes and is believed to cause damage to cells by injury to the membrane. Mechanisms for this injury include direct oxidation of membrane lipids by initiation of radical-mediated lipid peroxidation and by lowering of tissue pH (Mustafa and Tierney 1978). Because of its high reactivity and high solubility, NO_2 distribution is limited to the more proximal airspaces of the lungs, and its principal reactions occur with membranes of alveolar macrophages and epithelial cells. Because of its high reactivity and the focal nature of injury it produces, NO_2 is commonly referred to as the most toxic NO_x and is believed to be responsible for emphysematous lesions in the lungs. For instance, NO_2 was regarded as the critical toxic agent in the airspace enlargement and formation of alveolar pores in the lungs of beagle dogs chronically exposed to a combination of NO_2 and NO (Hyde et al. 1978). These results and other studies using higher concentrations have prompted some workers to suggest that NO_2 exposure results in emphysematous lesions. A number of studies, however, where NO was absent have demonstrated a fibrotic response for NO_2 in the alveolar region, with the most significant effects in bronchiole cells of the airways but no evidence for emphysematous lesion production (Johnson et al. 1982; Chang et al. 1986; Rombout et al. 1986). The role of NO in these early studies, which suggested that NO_2 produced emphysematous lesions, has not been defined.

Previously, my colleagues and I demonstrated that NO exposure produces fenestrae in alveolar septa (Mercer et al. 1995). These results suggest that the pattern of injury produced by NO might be significantly different from the increase in epithelial cell number and thickness and interstitial fibrosis seen with other inhaled oxidants. The extent of defects that the fenestrae produced in alveolar septa was small compared with the large surface area of the lungs. However, these changes suggested that higher levels of NO might have significant effects on the cells and connective tissues.

In this report we describe a morphometric study of the cells and connective tissue alterations due to NO exposure at 2 and 6 ppm for 6 weeks. These exposure regimes allow a direct comparison with previous morphometric studies of other oxidants. In addition, the concentrations of NO used in these exposures are significantly higher than in the previous study and would, therefore, be expected to enhance significantly the production of fenestrae if NO were an important causative agent.

For the study, an exposure to environmentally relevant concentrations of NO with negligible amounts of NO_2 was obtained by the use of pure source gases and high chamber-exchange rates. Morphometric methods were then used to determine precisely the specific cell types most sensitive to NO. Alveolar fenestrae were counted in serial sections to compare with the results from the prior study. In addition to identifying the parenchymal cells most sensitive to injury, we carried out a detailed study of alveolar architecture and extracellular matrix elements in order to compare the relative toxicity of each pollutant.

SPECIFIC AIMS

The focus of this study was to provide information on the response to inhaled nitric oxide that could be compared with the inhalation toxicity of nitrogen dioxide and other oxidants. To accomplish this, we focused on three Specific Aims.

SPECIFIC AIM 1

Conduct Measurements to Determine the Injury Induced by Nitric Oxide Exposure

In order to determine the injury to cells from NO, we utilized morphometric analyses of inflammatory responses and biochemical analyses of lavage fluid as sensitive indicators of cell injury and response.

SPECIFIC AIM 2

Identify the Parenchymal Cells Most Sensitive to Nitric Oxide Exposure

Epithelial cells are the most immediate and sensitive cell type affected by brief (hours to days) exposure to highly reactive oxidants such as NO_2 and O_3 (Chang et al. 1988). In order to compare responses to NO with responses to other oxidants, we conducted morphometric analyses to detect specific changes in epithelial and interstitial cells and in connective tissues, which are most frequently observed in exposure to NO_2 and O_3.

SPECIFIC AIM 3

Compare the Dose-Response for Nitric Oxide Inhalation with That of Other Oxidants

In this study we conducted exposures at 2 and 6 ppm to NO for a 6-week duration. These exposure concentrations and durations were designed to duplicate conditions present in prior studies of NO_2 (Chang et al. 1986) and thus provide a direct comparison of the dose-response between these two oxides of nitrogen.

STUDY DESIGN AND METHODS

EXPOSURE CONDITIONS

To achieve the goals of this study, it was important to choose concentrations and duration of exposure to inhaled NO that would be comparable with those previously reported for other oxidants. In prior studies of NO_2 toxicity, exposure concentrations between 0.5 and 10 ppm provided the most useful information on the critical location of injury, the cells most sensitive to injury, and environmentally relevant dose-response information (National Research Council 1986). Concentrations of 0.5 ppm, 2 ppm, and 6 ppm and exposure durations on the order of 6 weeks are commonly used in studies of pathology from exposure to NO_2 (Mustafa and Tierney 1978; Chang et al. 1986; National Research Council 1986; Rombout et al. 1986). Typically, concentrations greater than 6 ppm have been required to produce statistically significant endpoints for functional indicators such as bronchoalveolar lavage measurements. As we already had data from a previous study in the lower concentration range (0.5 ppm NO), we chose exposures of 2 and 6 ppm NO for 6 weeks to duplicate the concentrations used in prior studies of NO_2 and thus provide endpoints on the dose-response curve for comparisons between NO and NO_2. Ozone is recognized to be significantly more toxic than other common airborne oxidants such as NO_x. A previously published morphometric study of the same duration but employing a significantly lower concentration of O_3 (0.25 ppm) was used in comparisons (Chang et al. 1991).

All animals received food (Purina Rat Chow, Barnes Supply Co., Durham, NC) and water ad libitum. Animals were maintained on a 12-hour-on and 12-hour-off photoperiod at an average chamber temperature of 72°F and relative humidity of 39%. Animal use was in conformity with federal standards.

EXPERIMENTAL ENDPOINTS

In order to make the comparison of dose-response most useful, methods that had been previously used to study the toxicity of NO_2 (Chang et al. 1986, 1988) and O_3 (Barry and Crapo 1982; Barry et al. 1988; Chang et al. 1991) were used in this study. These techniques isolate the alveolar tissue from the proximal alveolar region, which corresponds to the alveoli immediately proximal to the terminal airways. This region was selected for study as this site is significantly more sensitive to injury from inhaled oxidants than are the more distal regions. These methods provided the following experimental endpoints:

1. Measurement of inflammatory cell number and volume in the lung was used as a sensitive indicator of lung response to injury from inhaled NO.
2. Cellular response to inhaled NO was identified by morphometric measurement of epithelial, interstitial, and endothelial cell number, surface area, and volume in the lung parenchyma.
3. Changes in connective tissue such as collagen and elastin fibers, basement membranes, and other noncellular interstitial matrix elements were monitored.
4. Bronchoalveolar lavage analysis of protein content provided an index of lung permeability change. Antioxidants such as ascorbic acid and the thiol antioxidant glutathione, which have the potential to limit the toxicity of NO, were monitored.

ANIMAL EXPOSURES

Specific pathogen–free male, F344 rats (CDF 9F-34401/CR 1 BR), 7 weeks old at the initiation of the exposures, were used. Three groups of rats, corresponding to 2 ppm NO, 6 ppm NO, and pollutant-free or clean-air exposures, were studied. Exposures were conducted in collaboration with the EPA using facilities, procedures, and equipment previously described (Crapo et al. 1984). Briefly, exposures were conducted in 0.323-m^3 stainless-steel chambers. In order to obtain exposures to pure NO and minimize contamination by NO degradation products such as NO_2, the chamber air was exchanged once every minute using a flow of 323 L/min of dry, filtered air directed into the top of each chamber. Gas-source tanks containing 10,000 ppm NO in nitrogen (N_2) were obtained from National Welders Gas Co. (Raleigh, NC). Contamination with NO_2 was less than 0.5% of the NO concentration. Nitric oxide from the gas-source tanks was metered by a mass-flow controller under computer control into the filtered air of the exposure chambers. For both the 2 and 6 ppm NO exposures, the concentrations of NO and of the potentially contaminating NO_2 were measured continuously via computer using the output from a chemiluminescence analyzer (Bendix Corporation Model 8101-C, Ronceverte, WV) calibrated according to EPA reference method RFNA-0777-022. Background O_3 was monitored with an O_3 analyzer (Bendix Corporation Model 8002, Ronceverte, WV), EPA reference method RFOA-0176-007. Computer-controlled feedback by means of a mass-flow controller was used to maintain the desired concentration. Exposures were reduced to zero for 2 hours each day to permit maintenance and animal care (10:00 a.m. to 12:00 noon). At alternating 5-minute intervals, the concentrations of NO_2 and O_3 were determined. The average residual NO_2 concentration was 0.03 ppm for the NO-exposed group and less than 0.001 ppm in the controls. Residual NO was not detectable in the control chamber. Residual O_3 in the control and NO chambers was less than 0.02 ppm.

Within 1 to 2 hours of the end of exposure, rats from the clean-air control and NO-exposed groups were killed and preserved for lung morphometry by transmission electron microscopy (TEM) (five per group) and for analyses of structural pathology by scanning electron microscopy (SEM) (five per group). Separate groups, corresponding to 2 ppm NO, 6 ppm NO, and pollutant-free or clean-air exposures, were studied using bronchoalveolar lavage (eight per group).

LUNG FIXATION

For lung fixation, each rat was anesthetized with intraperitoneal pentobarbital (75 mg/kg of body weight), and the trachea was cannulated. The abdominal cavity was opened, and both hemidiaphragms were punctured to deflate the lungs. The lungs were then fixed by inflation with 2% glutaraldehyde in an 0.085 M Na cacodylate buffer at a constant pressure of 20 cm above the midpoint of the chest wall. After fixation, the lungs were dissected free and the lung volume determined by displacement in fresh fixative (Scherle 1970). The right and left lungs were cut into approximately 2-mm-thick transverse slices. Four to five of these slices, uniformly sampling the lungs from top to bottom, were taken for paraffin embedding and subsequent determination of the parenchymal volume fraction. Four coronal slices from the upper and lower one-third of the left lung were taken for embedding and sectioning for TEM analysis. Adjacent slices from the right lung were critical-point dried and sputter coated by standard SEM methods.

TISSUE EMBEDDING, ISOLATION, AND SECTIONING

The tissue slices for TEM analysis were treated with 1% tannic acid (Gallotannin, Pfaltz & Bauer Inc, Waterbury, CT), adjusted to pH 7.4 and 475 mOsm with respect to sucrose, and washed with a cacodylate buffer. Tannic acid was included in the processing because it produces an electron-dense staining of connective tissue fibers in subsequent electron microscopy sections (Mercer and Crapo 1990). After fixation with osmium tetraoxide, the tissues were dehydrated in a graded series of alcohols and then transferred to propylene oxide. The dehydrated tissues were then passed through a graded series of epoxy embedding resin and propylene oxide and left in 100% embedding resin overnight. Following the overnight infiltration, the tissue was transferred to embedding molds and cured at 60°C for 16 to 24 hours. The proximal alveolar region was isolated from these tissues as previously described (Crapo et al. 1984). The embedded tissue block was softened with mild heat (40°C) and cut into slices approximately 0.3 mm thick. Each slice was examined with a dissecting microscope, and slices containing the air passage from the terminal bronchiole to the first alveolar duct bifurcation were selected. A 1-mm block containing tissue from this region was then cut from the slice and glued to a blank TEM block. The terminal bronchiole tissue was then shaved from the block, and serial sections (approximately 1 µm thick) were cut until the first alveolar duct bifurcation was reached. Excess tissue was trimmed away so that only tissue surrounding the first bifurcation and alveolar ducts remained. Ultrathin sections (60 nm) were picked up on 200-mesh copper grids. These sections, which typically covered 15 to 20 grid squares, were stained with uranyl acetate and lead citrate. For morphometric studies of the alveolar region, the tissue in the upper and lower

right grid squares was photographed and printed on 11" × 14" photographic paper at a final enlargement of × 8,500. A calibration grid was photographed with the set of micrographs from each section and used to ensure an accurate final magnification.

PROXIMAL ALVEOLAR REGION MORPHOMETRY

Stereologic procedures (Weibel 1980) specifically developed to study focal injuries in the proximal alveolar region were used to analyze the samples (Crapo et al. 1984).

The following formulas were used in the analysis of morphometric data:

$$V_V = P_i/P_T,$$

where V_V = volume density, P_i = the number of points on the tissue being studied, and P_T = the total number of points.

$$S_V = 2\ I_L,$$

where S_V = surface density (measured in μm^{-1}), and I_L = the number of intersections per test line length.

$$N_V = N_A/\overline{D},$$

where N_V = numerical density (designated as number $/mm^3$), N_A = number of cells per unit area of section (designated as number/mm^2), and $\overline{D}$ = mean caliper nuclear diameter.

$$t = V_V/S_V,$$

where t = arithmetic mean thickness (measured in µm). S_{BM} = alveolar epithelial basement-membrane surface area, and V_V/S_{BM} is measured in µm.

To determine the volume density (V_v) and surface density (S_v) of major tissue components, each electron micrograph was placed under a plastic overlay that contained 112 2-cm lines in a pattern described by Weibel (Weibel 1980). The end of each line was assumed to be a point, giving a total of 224 points for each micrograph. The points falling on each of the tissue components to be characterized were tallied for volume determination. Categories for the point-counting determination of volume density included type I epithelial cells, type II epithelial cells, interstitial cells, noncellular interstitium, endothelial cells, and alveolar macrophages. The noncellular interstitial matrix was further subdivided into components consisting of collagen fibers, elastic fibers, and other noncellular matrix elements such as basement membranes. The numbers of line intercepts with either an alveolar type I or type II cell on the epithelial surface, of line intercepts with the epithelial basement membrane, and of line intercepts with capillary endothelial surface were counted for determinations of alveolar surface area, alveolar epithelial basement-membrane surface area, and capillary surface area, respectively. The volume densities of blood elements (red blood cells, plasma, neutrophils, and other blood-borne cells) were determined in a separate counting of the same micrographs.

Samples for the above analysis were taken from the alveolar region immediately proximal to the terminal bronchiole as this region is more sensitive to injury from oxidants than are the more distal regions. Because the boundaries of the proximal alveolar region are difficult to define rigorously, volume and surface densities cannot be expressed as absolute values. To compare these results, the volume and surface densities were divided by the surface area of alveolar epithelial basement membrane in the same sample (volumes/S_{BM}). Variance that might result from different degrees of lung inflation would be reduced by this normalization procedure.

Arithmetic-mean thickness (t) of the epithelial, interstitial, and endothelial components in the proximal alveolar region was determined by dividing the volume density of the component by the respective surface density (Weibel 1980).

The number of cells for a given unit of alveolar epithelial basement-membrane surface in the proximal alveolar region was determined in order to assess the response of different cell types to the exposures (number/mm^2). This was accomplished according to previously published methods (Weibel 1980; Chang et al. 1986; Stone et al. 1992). Briefly, the frequency of occurrence of nuclear profiles/unit area was determined by counting the number of nuclear profiles of each class of parenchymal cells found in the grid squares for each section. The average area of grid spaces was determined using a low-magnification photograph of the grid used in each count. The number of cells per unit of alveolar epithelial basement-membrane surface area was determined by dividing the numerical density of each cell type by the surface density. The mean caliper diameter ($\overline{D}$) of cell nuclei was obtained from a prior study of control Fisher rats of the same age and weight (Stone et al. 1992). Cell categories consisted of alveolar type I and II epithelial cells, endothelial cells, interstitial cells, and macrophages with mean caliper diameters of 8.80, 7.11, 6.87, 7.26, and 7.43 µm, respectively. Previous studies have shown that even the severe injury caused by exposure to 85% oxygen resulted in changes of 11% or less in mean nuclear diameter for rat alveolar cells other than type II epithelial cells and a 21% increase in the nuclear diameter of type II epithelial cells (Crapo et al. 1978). In addition, prior studies of oxidant lung injury in which the level of injury was less showed no change in the mean caliper diameter of lung cells (Hayatdavoudi et al. 1981). As

the injury from NO observed here is low compared with that reported by Hayatdavoudi and colleagues (1981), we assumed that there were no significant changes in mean caliper diameter and used the same values for both the control and NO-exposed groups.

SERIAL SECTION ANALYSIS OF FENESTRAE

To analyze the frequency of fenestrae in alveolar septa, serial-section analysis was carried out on 3 blocks from each animal. From each block, 20 serial sections (0.5-µm thick) were cut, stained, and cover slipped for light microscopic photography. Every other section in the series was photographed and printed at a final magnification of × 100 on 11" × 14" photographic paper. The location of each fenestra or opening in the wall of the alveolar septa was marked on each series of prints, and the dissector technique (Sterio 1984) was used to count the number of defects in the volume of the series as previously described (Mercer et al. 1995). The total number of fenestrae for each lung was determined by multiplying the number of defects in each unit of parenchymal volume by the total parenchymal volume of each lung. The total parenchymal volume was obtained by multiplying the fixed-lung volume by the parenchymal volume fraction. Parenchymal volume fraction was measured by a point-count determination of the ratio of points on parenchymal tissue to the total number of points on the lung tissue in the paraffin-embedded lung sections for each group. Parenchymal tissue was defined as alveolar tissue and excluded bronchi, bronchioles, interlobular connective tissue, as well as blood vessels greater than 25 µm in diameter.

BRONCHOALVEOLAR LAVAGE

For analysis of lung lavage fluids, the animals were anesthetized with 5% halothane to a deep plane of anesthesia (Halothane Laboratories, Hackensack, NJ) and then killed by severing the abdominal aorta. Lungs were lavaged via the trachea with a single volume (35 mL/kg) of warm (37°C) saline solution, which was injected and withdrawn three times. After centrifugation to remove cells (400 × *g* for 15 minutes at 25°C), the cell-free supernatant was taken for analysis of ascorbic acid, glutathione, uric acid, and protein content using previously published methods. Ascorbic and uric acids were measured on 3% perchloric acid extracts by high-pressure liquid chromatography with electrochemical detection (Kutnink et al. 1985). Glutathione analysis (reduced plus oxidized) employed enzymatic recycling (Anderson 1985). Protein was assayed by Coomassie blue using bovine serum albumin as a standard (Kodavanti et al. 1995).

STATISTICAL METHODS AND DATA ANALYSIS

Analysis of variance (ANOVA) was used to evaluate the significance between measurements. Data were tested for homogeneity of variance prior to ANOVA and normalized as appropriate. Significant ANOVA effects were subtested with Duncan's multiple-comparison test (Duncan 1955). All tests were two-sided tests, and $p < 0.05$ was considered to be significant. Data are given as means ± SE. The number of animals used in the test is indicated by *n*.

RESULTS

GROSS EFFECTS

Results of body weights for each study group are given in Table 1. No significant changes were found in body weights. No adverse effects on or mortality of animals were observed during exposure.

BRONCHOALVEOLAR LAVAGE

The alveolar fluid content of potential mediators of nitric oxide toxicity was examined by analysis of glutathione, ascorbic acid, and uric acid in bronchoalveolar lavage (Figure 1). The glutathione and ascorbic acid content of bronchoalveolar lavage was not significantly altered by NO exposure. The uric-acid content of the lavage fluid from the 6 ppm NO-exposure group was increased approximately 56% above that from the group control. Because relatively little is known about the role of uric acid as an antioxidant, the significance of this increase is uncertain. No significant difference was found in the total lavage protein content, which was 1.1 ± 0.3,

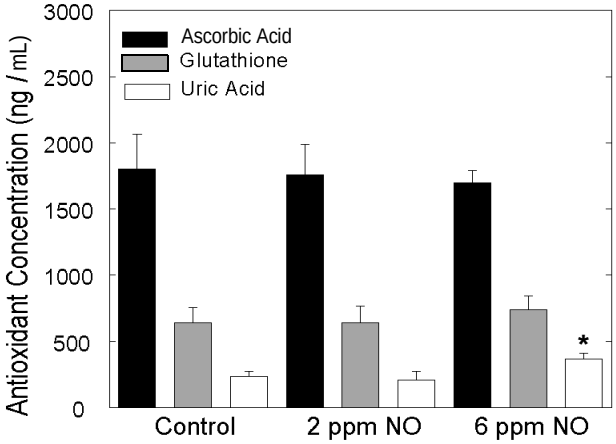

Figure 1. Comparison of bronchoalveolar lavage antioxidant content for control and NO-exposed rats. Data are means ± SE for 8 animals per group. *Significantly different from control rats ($p < 0.05$).

Table 1. Parameters Determined for Control Rats and Rats Exposed to 2 or 6 ppm NO for 6 Weeks[a]

Parameter	Control	2 ppm NO	6 ppm NO
n	5	5	5
Body weight (g)	274.2 ± 7.5	264.9 ± 7.6	270.5 ± 6.7
Fixed lung volume (mL)	6.71 ± 0.32	5.89 ± 0.57	6.22 ± 0.22
Basement-membrane surface density (cm^{-1})	335 ± 28	356 ± 21	331 ± 13
Volumes/S_{BM} (μm)			
Air	28.487 ± 2.216	26.630 ± 1.660	28.699 ± 1.296
Capillary lumen	1.141 ± 0.228	0.835 ± 0.195	0.805 ± 0.180
Tissue	0.892 ± 0.037	0.961 ± 0.110	0.918 ± 0.040
Type I epithelium	0.166 ± 0.005	0.173 ± 0.018	0.163 ± 0.013
Type II epithelium	0.073 ± 0.013	0.100 ± 0.007	0.103 ± 0.007
Cellular interstitium	0.174 ± 0.014	0.194 ± 0.035	0.180 ± 0.021
Interstitial collagen fibers	0.106 ± 0.009	0.126 ± 0.016	0.118 ± 0.096
Interstitial elastin fibers	0.035 ± 0.005	0.030 ± 0.007	0.028 ± 0.003
Other noncellular interstitium	0.090 ± 0.006	0.069 ± 0.008	0.063 ± 0.011
Endothelium	0.226 ± 0.011	0.237 ± 0.025	0.224 ± 0.008
Airspace macrophages	0.014 ± 0.001	0.026 ± 0.012	0.030 ± 0.004
Surface area (%)			
Type I epithelium (%)	98.3 ± 0.1	96.8 ± 0.6[b]	96.6 ± 0.2[b]
Type II epithelium (%)	1.7 ± 0.1	3.2 ± 0.6[b]	3.4 ± 0.2[b]
Arithmetic mean thickness (μm)			
Epithelium	0.239 ± 0.017	0.273 ± 0.022	0.266 ± 0.015
Type I epithelium	0.164 ± 0.004	0.173 ± 0.017	0.163 ± 0.014
Type II epithelium	4.172 ± 0.697	3.632 ± 0.803	2.931 ± 0.241
Interstitium	0.437 ± 0.029	0.460 ± 0.071	0.438 ± 0.042
Endothelium	0.254 ± 0.018	0.282 ± 0.038	0.282 ± 0.039

[a] All data are means ± SE.

[b] Significantly different from control group ($p < 0.05$).

0.73 ± 0.1, and 0.80 ± 0.1 mg/mL of lavage fluid in the control group and the 2 ppm and 6 ppm NO-exposed groups, respectively (means ± SE, $n = 8$).

QUANTITATIVE MORPHOLOGY

Results of the morphometric analyses are summarized in Tables 1 and 2. Body weights and lung volumes of clean air control and NO-exposed rats were not significantly different. Likewise, the surface density of the alveolar basement membrane was not affected by NO exposure. The average thickness of epithelial, interstitial, and endothelial compartments of the alveolar septum are given in Table 1. The average thickness of the type II alveolar epithelium, although reduced in NO-exposed lungs, was not statistically different from that in the control group lungs.

Morphometric analysis of the alveolar region demonstrated significant responses of both resident lung cells and inflammatory cells to inhaled NO. A response by alveolar type II cells was evidenced by the significantly greater fraction of the alveolar surface covered by this cell type in both 2 and 6 ppm NO-exposed lungs and by a 52% increase in type II cell number/mm^2 of basement-membrane surface area in the 6 ppm NO-exposed group. There was an approximate threefold increase in the number of alveolar macrophages in the airspaces of rat lungs exposed to 6 ppm NO (Table 2). The average number of alveolar macrophages in the airspaces of 2 ppm NO-exposed lungs, although elevated, was not statistically different from that in the control group.

As illustrated in the transmission-electron micrograph, Figure 2, inhaled NO produced significant sequestration

Table 2. Morphometrically Determined Characteristics of Parenchymal Cells in the Proximal Alveolar Region of Control Rats and Rats Exposed to 2 or 6 ppm NO for 6 Weeks[a]

Parameter	Control	2 ppm NO	6 ppm NO
n	5	5	5
Total number of cells/mm^2	1807 ± 110	2135 ± 116	2276 ± 209
Alveolar type I cells			
Number/mm^2	141 ± 11	171 ± 13	123 ± 17
% Total lung cells	8	8	5
Alveolar type II cells			
Number/mm^2	215 ± 17	278 ± 16	328 ± 43[b]
% Total lung cells	12	13	14
Endothelial cells			
Number/mm^2	903 ± 70	1045 ± 54	1073 ± 77
% Total lung cells	50	49	47
Interstitial cells			
Number/mm^2	509 ± 67	565 ± 76	624 ± 81
% Total lung cells	28	26	28
Airspace macrophages			
Number/mm^2	39 ± 7	72 ± 8	122 ± 18[b]
% Total lung cells	2	3	5

[a] All data are means ± SE.

[b] Significantly different from control group ($p < 0.05$).

of platelets in pulmonary capillaries. The volume density of platelets in the pulmonary capillaries was increased approximately twofold in the NO-exposed groups, being 0.0136 ± 0.002, 0.0286 ± 0.004, and 0.023 ± 0.002 in the control group and the 2 ppm and 6 ppm NO-exposed groups, respectively (mean ± SE, $n = 5$). Although present in higher numbers, the platelets did not demonstrate morphologic features of activation such as large, irregular profiles. No significant differences were found in the blood-element volume density of neutrophils or other circulating cells.

LUNG FENESTRAE

Results from counting lung fenestrae in serial sections are given in Table 3. There were no statistically significant differences in the total lung fenestrae among groups. (In our prior study [Mercer et al. 1995], the average numbers of total lung fenestrae were 9 [± 9] × 10^3 and 323 [± 156] × 10^3 for control and NO exposure groups, respectively.) Parenchymal volume fraction was 0.81 ± 0.01, 0.82 ± 0.02, and 0.81 ± 0.02 in the control group and the 2 and 6 ppm NO-exposed groups, respectively. There were no significant differences in parenchymal volume fraction among groups.

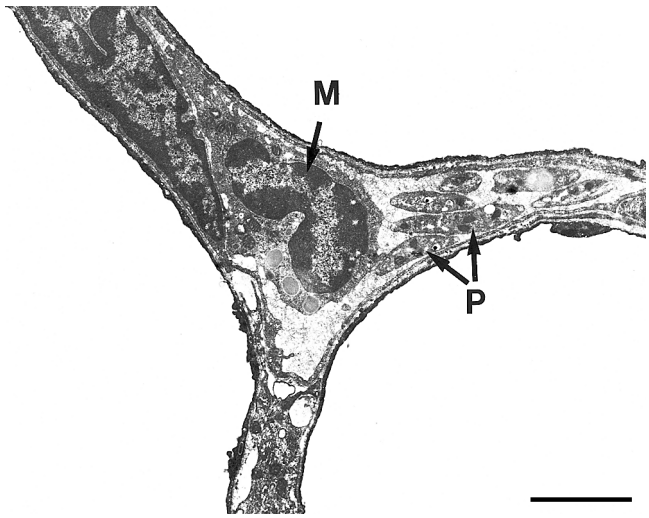

Figure 2. Transmission-electron micrograph demonstrating sequestration of platelets (P) in the capillaries of a rat lung exposed to 2 ppm NO. A circulating monocyte is also present in the capillary profile (M). Bar = 2 µm.

Table 3. Total Lung Fenestrae Determined by Serial-Section Analysis of Control Rats and Rats Exposed to 2 or 6 ppm NO for 6 Weeks[a]

Parameter	Control	2 ppm NO	6 ppm NO
n	5	5	5
Number/lung (×1,000)[b]	23.0 ± 4	55 ± 18	29 ± 5

[a] All data are means ± SE.

[b] No statistically significant differences were noted among groups ($p < 0.05$ by Duncan's multiple-comparison test).

SCANNING ELECTRON MICROSCOPE EVALUATION

Scanning electron microscopy was used to visualize fenestrae in the lungs in coronal slices of the right lobe. Under SEM examination, fenestrae were found to be distributed throughout the gas-exchange region of the lungs. Fenestrae could also be detected in control lungs, however, and as demonstrated by quantitative analysis, there was no significant difference in the number of fenestrae between control and NO-exposed lungs.

COMPARISON OF NITRIC OXIDE RESPONSE WITH THAT OF OTHER OXIDANTS

In order to compare the effects of NO with those of other oxidants, data from prior morphometric studies comparing the effects of O_3 and NO_2 in the proximal alveolar region were used (Crapo et al. 1984). The O_3 and NO_2 experiments in the referenced study were not done concurrently and thus have separate control groups of animals for each exposure. Exposures were conducted in the same facilities as described for the NO exposures in this report. Ozone was generated from 100% oxygen with an Orec Model 03V5-0 Ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ); mixed with dry, filtered air; directed into the top of the chambers; and monitored with a chemiluminescence ozone analyzer, EPA reference method RFOA-0176-007 (Bendix Corporation Model 8002, Ronceverte, WV). A chamber flow of 323 L/min NO_2 was achieved by adding a mixture of 1% NO_2 in N_2 to air flowing through the chamber at the same rate as in the O_3 study. NO_2 exposures involved the same monitoring as that described for the NO-exposure studies, where the NO concentration was maintained at 0.25 ± 0.01 ppm (mean ± SE) for 12 hours/day, 7 days/week for 6 weeks. The NO_2 concentration was maintained at 2.0 ± 0.02 ppm for 23 hours/day, 7 days/week, for 6 weeks.

The comparison among these three oxidants (Figures 3 and 4) shows a similar pattern of increased cell number for epithelial cells and alveolar macrophages as well as an increase in the percentage of epithelial basement membrane surface area covered by type II epithelial cells. Differences in the magnitude of the changes account for the most significant difference in lung cell responses to low-level exposures of different oxidants. For instance, O_3 produced a much greater increase in type I epithelial cell number than did either NO_x or NO_2, and NO produced a greater coverage of the alveolar surface by type II epithelial cells than did O_3.

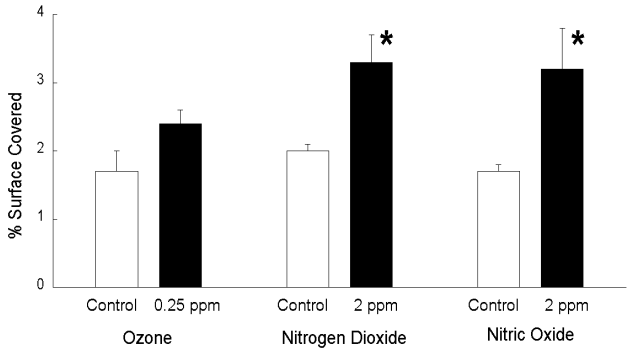

Figure 3. Comparison of the percentage of alveolar epithelial surface area covered by type II epithelial cells exposed to O_3, NO_2, or NO. Data are means ± SE; $n = 8$ for O_3, $n = 6$ for NO_2, and $n = 5$ for NO. *Significantly different from control group by Duncan's multiple-comparison test ($p < 0.05$). Data for O_3 and NO_2 are from previously reported studies described in the Results section.

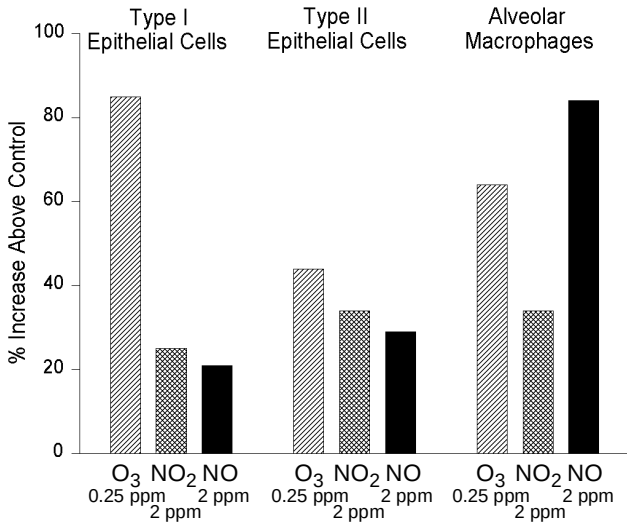

Figure 4. Percentage of change in the number of cells in alveolar septa from the proximal alveolar region after exposure to low doses of O_3, NO_2, or NO. Data are means ± SE; $n = 8$ for O_3, $n = 6$ for NO_2, and $n = 5$ for NO. Data for O_3 and NO_2 are from previously reported studies described in the Results section.

DISCUSSION AND CONCLUSIONS

EFFECTS ON PLATELET AGGREGATION

In this study of subacutely NO-exposed lungs, we qualitatively demonstrated an increase in platelet aggregation and quantitatively an increase in platelet-volume density in the pulmonary capillaries (see the Results section and Figure 2). This result is surprising as short-term exposures (hours) to concentrations of NO comparable to those used in this study are known to prevent platelet aggregation by NO-enhanced production of guanosine 3′:5′-cyclic monophosphate (cGMP) (Alheid et al. 1987). A biphasic response for inhaled NO, however, has also been reported in studies of immune function where acute exposure to inhaled NO produces an enhanced immune response, and chronic exposure produces a suppressed immune response (Holt et al. 1979). Habituation to inhaled NO production of cGMP would be a likely cause for a biphasic response in platelet aggregation. In a recent study of cyclic nucleotides in the lung, it was found that inhaled NO increased the amount of cGMP for the first 24 hours of exposure, but with continued exposure for 1 week, the amount of cGMP returned to that present in the control sample (Mercer 1997).

EFFECTS ON LAVAGE PROTEIN AND ANTIOXIDANTS

Antioxidants that are present in the liquid-lining layer of the lungs are likely to play an important role in scavenging superoxide because superoxide plays a critical role in the degradation of NO. Nitric oxide also reacts with thiols to form nitroso compounds, which are bioactive forms of NO and have a significantly longer half-life than NO (Stamler et al. 1992). Thus, antioxidants and thiols have the potential to alter significantly the toxic and beneficial effects of NO. The thiol and antioxidant glutathione, as well as ascorbic acid, are major antioxidants present in the lungs and play an essential role in protecting the lungs from oxidant pollutants (Cross et al. 1994). Changes in the amounts of these antioxidants present in lavage fluids have been shown to be sensitive indicators of damage by oxidants such as NO_2 (Massaro et al. 1988). The finding that the glutathione and ascorbic acid content of the lavage fluid is normal and the absence of changes in lavage protein suggest that inhaled NO does not produce a significant oxidant stress.

COMPARISON WITH PREVIOUS NITRIC OXIDE STUDIES

As indicated in the Introduction, the starting basis for the current study was a prior morphometric study of rats exposed for 9 weeks to 0.5 ppm NO, which demonstrated focal degeneration of the alveolar interstitium and increased numbers of fenestrated alveolar septa (Mercer et al. 1995). Several studies prior to ours had suggested that inhaled NO, or NO in combination with NO_2, produced an increase in alveolar fenestrae (Azoulay et al. 1977; Holt 1979; Hyde et al. 1978). Given the different NO_x concentrations and duration of exposure as well as the animal species used in these studies, detection of an apparent pattern is unlikely. The limited size and distribution of the defects observed in the prior NO exposure did not alter the alveolar surface area or other morphometric indicators of lung function but suggested that an exposure of higher NO concentration and longer duration would shed additional light on the findings.

In examining lungs exposed to concentrations of 2 and 6 ppm NO, we have found no evidence for changes in alveolar fenestration or interstitial composition. These results indicate that the changes noted in the prior study, which incorporated NO at a lower concentration, did not demonstrate a significant difference between NO-exposed and control groups. Although this conclusion is straightforward, a number of factors may complicate this interpretation.

Given recent findings on the concentration dependence of NO action, it is not unexpected that studies differing in NO concentration and exposure duration would produce contradictory findings. Nitric oxide is an agent used by inflammatory cells to kill bacteria, an anti-inflammatory agent (which prevents platelet aggregation), and a physiologic modulator of vascular tone. In recent years it has become apparent that the action of NO may be either beneficial or injurious, depending on the concentration of NO and the oxidant stress level. As discussed in a review of the NO concentration-dependent kinetics (Beckman and Koppenol 1996), NO has three principal reactions. Under physiologic conditions, both the concentration of NO and of superoxide are low. The half-life of NO is relatively long, and NO acts during vasodilation by binding to guanylate cyclase or is eliminated by binding to oxyhemoglobin. At higher concentrations of NO or superoxide, the concentration-dependent reaction kinetics favor the formation of toxic oxidants. These and related concentration-dependent effects of NO in lipid peroxidation (Rubbo et al. 1994) indicate that NO dose-responses may be complex under a wide range of exposure concentrations and durations.

RESPONSES TO NITRIC OXIDE, NITROGEN DIOXIDE, AND OZONE

The increased basement-membrane coverage by type II epithelial cells and increased number of type II epithelial cells and airspace macrophages observed at an exposure concentration of 6 ppm are consistent with the NO action to form toxic oxidant products. This proinflammatory action is further supported by the apparent aggregation of platelets in alveolar capillaries. Not all of the data are consistent with this pattern, however. Presumably, this action of NO would enhance the lung's oxidant stress and would also produce responses in antioxidants such as ascorbic acid. Such changes are known to occur for other oxidants but were not apparent in the NO study. Changes in the connective tissue matrix and interstitial cells are major responses in chronic exposure to oxidants. No noteworthy changes in the interstitial cell or connective tissue compartment were identified in the responses to inhaled NO.

SUMMARY

Rats were exposed for 6 weeks to 2 and 6 ppm NO inhaled under conditions where contamination by NO_2 was minimized. Following exposure, the lungs were preserved and analyzed by morphometric methods that were specifically developed to detect injury from inhaled oxidants. Significant responses included an increase in the fraction of the epithelial basement membrane covered by type II epithelial cells and an increase in the number of type II epithelial cells and alveolar macrophages. Although alveolar fenestrae were present in NO-exposed lungs, the number of fenestrae was not significantly different between the control and exposed groups. The results demonstrate that inhaled NO produces noticeable proinflammatory activation of the lungs in a pattern similar to that of other oxidants. These data would indicate that environmental exposures to NO that are typically less than 0.1 ppm do not pose a potential health hazard.

IMPLICATION OF FINDINGS

This study indicates that NO has a pattern of response similar to that of other oxidants, which would suggest that methods used to evaluate health risks for other oxidants would also be appropriate for NO.

As is the case for all oxidants that recruit inflammatory cells into the lungs, the potentially "primed" population of lung inflammatory cells recruited by inhaled NO may produce injurious responses during coexposure to other toxicants, such as particles, which invoke a response by alveolar macrophages.

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ABOUT THE AUTHOR

Robert R. Mercer received a Ph.D. in biomedical engineering in 1982 from the University of North Carolina. His postdoctoral study (1983–1987) investigating structural changes in lung injury was completed at the Department of Medicine at Duke University. Upon completion of the fellowship in 1987, he joined the faculty at Duke. In 1997, he joined the Physiology and Pathology Research Branch at the National Institute of Occupational Safety and Health, Morgantown, WV. His research interests include methods of quantitative analysis of biologic structure, lung micromechanics, and the application of these methods to the study of connective tissue disorders due to inhaled toxicants and pulmonary diseases.

PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS

ANOVA	analysis of variance
cGMP	guanosine 3′:5′-cyclic monophosphate
$\overline{D}$	mean caliper diameter of cell nucleus
I_L	number of intersections per test line length
mOSM	milliosmolal
N_2	nitrogen
N_A	number of cells per unit area of section
NAAQS	National Ambient Air Quality Standard
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	oxides of nitrogen
N_V	numerical density (number of cells per unit volume of lung parenchyma)
O_3	ozone

P_i	number of points on tissue being studied
P_T	total number of points
S_{BM}	alveolar epithelial basement-membrane surface area
SEM	scanning electron microscopy
S_V	surface density of major tissue components
t	arithmetic mean thickness
TEM	transmission electron microscopy
volumes/S_{BM}	volume and surface densities divided by alveolar epithelial basement-membrane surface area
V_V	volume density of major tissue components

INTRODUCTION

Nitric oxide (NO)* is formed by several combustion processes, including those of fossil fuels and tobacco products. Outdoor atmospheric concentrations of NO may reach as high as 0.12 to 0.16 parts per million (ppm), and indoor concentrations generally average 0.11 ppm (Schlesinger 1992). However, indoor concentrations in homes where gas is used for cooking or where tobacco smoke is present can reach 0.4 ppm (Schlesinger 1992); levels of NO in tobacco smoke may reach as high as 1,000 ppm (National Research Council 1986). Because NO is easily converted to nitrogen dioxide (NO_2) in ambient air, little attention has been directed toward the toxicity of NO at ambient concentrations and no National Ambient Air Quality Standard (NAAQS) for environmental levels has been established. The occupational standard for exposure set by the Occupational Health and Safety Administration is 25 ppm as an eight-hour time-weighted average (National Institute of Occupational Safety and Health 1976).

Nitric oxide is both toxic and beneficial to humans. When inhaled, NO can be converted to NO_2 (as one reaction under physiologic conditions), or to peroxynitrite and peroxynitrous acid, both of which are powerful oxidizing agents that can facilitate lipid peroxidation and damage cells and tissues (Gaston et al. 1994). Although inhalation of NO can produce toxic effects, it is also produced endogenously through a reaction involving NO synthetase and arginine and it has a role as a physiological mediator. Endogenous NO modulates several processes, including relaxation of smooth muscle, inhibition of platelet aggregation, stimulation of hormonal release, bacterial killing, autonomic and central neurotransmission, and tumor cell lysis (Gaston et al. 1994). Nitric oxide inhalation is being used increasingly in medical applications and has been used recently to treat adult respiratory distress syndrome (Rossaint et al. 1993) and pulmonary hypertension of the newborn (Pepke-Zaba et al. 1991; Gaston and Stamler 1997).

In spite of its physiologic role and its use as a therapeutic agent, the toxicity of NO has not been extensively studied, in part because of the technical difficulties inherent in generating pure NO for studies of inhalation toxicity. Most studies of the effects of ambient oxides of nitrogen have focused on NO_2. In 1995, Dr. Mercer and his coworkers reported a study of lung ultrastructure to compare the effects of pure NO and pure NO_2. To achieve the proper exposures, they used pure source gases combined with high chamber exchange rates to eliminate the possibility of gas conversion (Mercer et al. 1995). For nine weeks, the investigators exposed rats to 0.5 ppm with twice-daily one-hour spikes of 1.5 ppm of either NO or NO_2. They reported several effects that appeared to be induced by NO, including a reduction in the thickness of interstitial components of the alveolar wall, and small reductions in type I epithelial cells and endothelial cells. Most significant, however, were the findings of holes or fenestrae in the alveolar septa in the exposed animals. Although present in animals in both exposure groups, Mercer and colleagues found more and larger fenestrae in the animals exposed to NO. The number of fenestrae in the control group of animals was not significantly different from zero. Overall it appeared that NO led to destruction of lung tissue.

In 1994, Dr. Mercer submitted a preliminary application to HEI seeking funds to study pulmonary lesions in tissues from rats exposed to two concentrations of NO (2 ppm and 6 ppm) and to compare these effects to those produced by two oxidant air pollutants, ozone and NO_2. The Research Committee supported this research because NO is present in motor vehicle emissions and information on its toxicity is limited. Dr. Mercer then submitted a full application that the Research Committee recommended for funding.†

TECHNICAL EVALUATION

The investigator's specific aims were to determine:

1. the location of injury caused by NO or its reaction products, the cells most sensitive to NO, and the pathologic and functional changes induced by NO;
2. the time course of NO-induced injury and whether it is mediated or amplified by inflammatory cells; and

* A list of abbreviations appears at the end of the Investigator's Report for your reference.

† Dr. Mercer's two-year study, *Morphometric Analysis of Alveolar Responses of F344 Rats to Subchronic Inhalation of Nitric Oxide*, began in February 1995 and had total expenditures of $195,170. The Investigator's Report was received for review in January 1998. A revised report, received in September 1998, was accepted for publication in October 1998. During the review process, the HEI Review Committee and the investigator exchanged comments and clarified issues in the Investigator's Report and in the Review Committee's Commentary.

3. the differences and similarities between NO-induced responses and those from other oxidant gases, especially ozone and NO_2.

To accomplish these objectives, Dr. Mercer continuously exposed 7-week-old male rats (18 animals per group) to 2 or 6 ppm NO or to air for 6 weeks, except for two hours daily from 10 a.m. to noon to allow time for maintenance and animal care. To achieve negligible levels of NO_2, a pure source of gas was used and air exchange rates in the chambers were high. The average contaminating concentration of NO_2 was 0.03 ppm and of ozone was less than 0.02 ppm.

The investigator performed bronchoalveolar lavage on eight animals per group. Lavage analyses included measurements of markers of lung permeability (protein) and levels of antioxidants such as ascorbic acid and glutathione. He also measured the uric acid content of lavage fluid. At the termination of the exposure period, the investigator killed an additional five animals from each group, and fixed their lungs under standardized conditions for morphometric analysis using transmission electron microscopy. The remaining animals were killed and their tissues were examined by scanning electron microscopy. End points included:

1. quantitative changes in the structure of connective tissues (collagen and elastin), epithelial and endothelial membranes, and other noncellular interstitial matrix elements;
2. changes in alveolar structure, including surface area, size, and number and type of abnormalities in alveolar septa; and
3. lavage fluid indicators of lung injury (total protein, albumen, cell count, elastase activity, desmosine, hydroxyproline, and NO scavengers).

Dr. Mercer used rigorous means to ensure precise exposure to NO without cross-contamination by other oxidants, especially NO_2. He successfully obtained the proposed morphometric measurements of alveolar tissue compartments and numbers of various cells in the alveoli (airspace macrophages, type I and type II epithelial cells, interstitial cells, and platelet aggregates) and analyzed lavage fluid for protein and several antioxidants (ascorbic acid, glutathione, and uric acid). He did not report the time course of injury from exposure to NO, nor did he clarify whether the injury was mediated or amplified by inflammatory cells. Although studies looking at the effects of ozone and NO_2 were not conducted concurrently, these comparisons have been carefully conducted by the investigator and provide useful information for comparing the health effects of all three oxidants.

The state-of-the-art histologic and morphometric methods used by the investigator are a strength of the study. The procedures for lung fixation, sectioning, and microscopic analysis conformed to accepted methods, as did those used to analyze lavage fluid for total protein, glutathione, ascorbic acid, and uric acid.

Analysis of variance was used to evaluate statistical significance among measurements. Once significance was seen, Duncan's (1955) multiple-range test was used. The tests were two-sided and $p < 0.05$ was considered to be significant. This procedure is appropriate here because more than two groups were tested for differences in the means.

RESULTS AND INTERPRETATION

In the animals exposed to 2 or 6 ppm NO (compared with control animals), Dr. Mercer found an increase in the alveolar surface area covered by type II cells, increased numbers of alveolar macrophages, and platelet aggregates in the alveolar microvessels. These changes are consistent with findings in earlier studies of the effects of NO, NO_2, and ozone using the same experimental protocol. However, the exposure concentrations for NO and NO_2 are much higher than typical ambient levels, which generally range from 0.03 to 0.05 ppm for NO_2 (U.S. Environmental Protection Agency 1999) and 0.01 to 0.12 ppm for NO (National Research Council 1986).

The investigator found no increase in alveolar fenestrae in the NO-exposed animals compared with control animals, and NO exposure did not cause a statistically significant alteration in either the thickness of individual alveolar wall compartments or the entire wall, although focal areas of "interstitial degeneration" were apparent. No differences were noted in the levels of total protein, ascorbic acid, or glutathione in the lavage fluid of control and NO-exposed animals. The uric acid concentration in lavage fluid from animals exposed to 6 ppm NO was 56% higher than in fluid from control animals. The meaning of this observation is uncertain.

The major findings of this study differ from those previously reported by the same investigator. These discrepancies are particularly perplexing because the concentration of NO in the previous study (0.5 ppm with twice-daily spikes of 1.5 ppm) was much lower than that used in the current HEI study. (Animals were the same age in both studies.) In his previous study (Mercer et al. 1995), Dr. Mercer reported 328×10^3 fenestrae per lung in the NO-exposed group, 99×10^3 per lung in the NO_2-exposed

group, and 9×10^3 per lung in the control group (not significantly different from zero).

The earlier study also ascertained that the thickness of the alveolar interstitium and the number of interstitial cells decreased in response to NO exposure, whereas the present study found that these measurements did not change as a result of NO exposure. Direct comparisons of some of the findings between the two studies are complicated by the fact that Dr. Mercer used different units of measure for alveolar volume in his two publications. Futhermore, it is not clear whether alveolar sampling was done in the same way in both studies.

SUMMARY

Nitric oxide is an air pollutant and is gaining use as a therapeutic agent. The report describes a careful morphometric study of the effects on the rodent lung of controlled exposure to NO free from contamination with other oxides of nitrogen. This study is important because it is one of few morphometric analyses of the effects of pure NO and adds to the limited data on NO toxicity. The changes in alveoli resulting from exposure to NO appear to be similar to those resulting from exposure to ozone or NO_2. In contrast to Dr. Mercer's earlier study (Mercer et al. 1995), however, no significant increases in alveolar fenestrae or decreases in alveolar interstitium or the number of interstitial cells were found in this study. The discrepancy between findings of increased fenestrae in Dr. Mercer's previous study (Mercer et al. 1995) and no fenestrae increase in the present study is a major unaddressed issue and possible explanations should be explored. Despite these disparities, this study provides the first detailed description of effects of NO on lung structure and serves as a basis for future studies of this pollutant.

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