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Aldehydes (Nonanal and Hexanal) in Rat and Human Bronchoalveolar Lavage Fluid After Ozone Exposure

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 90
November 1999

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HEI Statement

Synopsis of Research Report Number 90

A Pilot Study of Potential Biomarkers of Ozone Exposure

BACKGROUND

Ozone, a major constituent of smog and a lung airway irritant, induces transient declines in lung function and respiratory tract inflammation in some people. Studies with laboratory animals have demonstrated that pathologic and physiologic effects of ozone on the respiratory system depend on the dose and duration of exposure. Although sensitive and accurate methods are available to measure the levels of ozone in ambient air, no methods have been developed to determine the dose of ozone that reaches tissues in the respiratory tract. Such methods would aid researchers conducting clinical studies and those seeking to extrapolate the results of animal studies to humans. Some inhaled pollutants react with tissue constituents to form products that can be measured in blood, urine, or other fluids and reflect the dose received by a tissue. These products are referred to as *biomarkers of dose*. No biomarkers for ozone exposure have been identified. Ozone is a highly reactive gas and is unlikely to penetrate far beyond the fluid that lines the lung's epithelial cell layer. Ozone's harmful effects are thought to be mediated by products of its reaction with components of the lining fluid and the epithelial cell membrane. These products include aldehydes which, although rapidly metabolized, can be toxic to cells. Thus, the levels of aldehydes in lung fluids may serve as biomarkers of the dose of ozone received by the lung.

HEI supported Dr. William A. Pryor of Louisiana State University to develop methods for measuring ozone reaction products in in vitro models of lung lining fluids exposed to ozone and in lung fluids from rats exposed to ozone. During the study, Dr. Mark Frampton of the University of Rochester provided Pryor with lung fluids from humans exposed to air or ozone under controlled conditions. In the current pilot study, Pryor and colleagues analyzed these fluids for two aldehydes that are known to be ozone reaction products. This report describes the results of the collaborative study between Drs. Pryor and Frampton.

APPROACH

In an earlier study, Frampton and colleagues exposed exercising smokers and nonsmokers to filtered air or to 0.22 parts per million (ppm) ozone for four hours. They obtained lung fluid samples from the subjects either immediately after or 18 hours after exposure ended. Pryor and collaborators analyzed the samples for two aldehydes, nonanal and hexanal. These aldehydes are formed by ozone reacting with unsaturated fatty acids found in the lung lining fluid and cell membranes.

RESULTS AND IMPLICATIONS

The investigators reported that nonanal levels were significantly higher in lung fluid samples obtained immediately after ozone exposure ceased and returned to control levels (established from exposures to filtered air) 18 hours after exposure ceased. (Changes in hexanal were not statistically significant at either time point.) Smokers and nonsmokers showed similar increases.

The increased level of nonanal suggests that aldehydes may be useful markers of ozone exposure. (Nonanal is also a toxic compound that may play a role in the adverse effects caused by ozone exposure.) However, aldehyde identification was not rigorously quantified in this study; therefore, the results must be considered as qualitative rather than quantitative. In addition, because the subjects in this study were exposed to only one concentration of ozone, studies using a range of ozone levels are required to confirm this preliminary observation and substantiate the relation between nonanal formation and ozone exposure level.

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 in 1994. This study provides the results of a collaboration between Drs. Mark W. Frampton of the University of Rochester School of Medicine and William A. Pryor of Louisiana State University. The following Research Report contains both the detailed Investigators' Report and a Critique on the study prepared by the Institute's Health Review Committee.

Library of Congress Catalog Number for the HEI Report Series: WA 754 R432

The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25, 26, 32, 51, and 65 Parts IV, VIII, and IX excepted. These excepted Reports are printed on acid-free coated paper.

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INVESTIGATORS' REPORT

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ABSTRACT

We hypothesized that exposure of healthy humans to ozone at concentrations found in ambient air causes both ozonation and peroxidation of lipids in lung epithelial lining fluid. Smokers (12) and nonsmokers (15) were exposed once to air and twice to 0.22 ppm ozone for four hours with exercise in an environmental chamber; each exposure was separated by at least three weeks. Bronchoalveolar lavage (BAL)* was performed immediately after one ozone exposure and 18 hours after the other ozone exposure. Lavage fluid was analyzed for two aldehyde products of ozonation and lipid peroxidation, nonanal and hexanal, as well as for total protein, albumin, and immunoglobulin M (IgM) as markers of changes in epithelial permeability. Ozone exposure resulted in a significant early increase in nonanal ($p < 0.0001$), with no statistically significant relationship between increases in nonanal and lung function changes, airway inflammation, or changes in epithelial permeability. Increases in hexanal levels were not statistically significant ($p = 0.16$). Both nonanal and hexanal levels returned to baseline by 18 hours after exposure. These studies confirm that exposure to ozone with exercise at concentrations relevant to urban outdoor air results in ozonation of lipids in the airway epithelial lining fluid of humans.

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

This Investigators' Report is one part of Health Effects Institute Research Report Number 90, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Mark W. Frampton, University of Rochester School of Medicine, 601 Elmwood Avenue, Box 692, Rochester, NY 14642-8692.

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.

INTRODUCTION

Ozone, a strong oxidant, is a toxic air pollutant produced from photochemical processes acting on precursor pollutants. Exposure to ozone, particularly with exercise, is associated with respiratory symptoms, decrements in lung function, airway injury and inflammation, limited athletic performance, exacerbation of asthma, and increased mortality (Bascom et al. 1996). Although the U.S. Environmental Protection Agency (EPA) has promulgated a new, more stringent air quality standard for ozone, ambient concentrations in many U.S. cities regularly exceed the previous one-hour standard of 0.12 ppm (Bascom et al. 1996). Achieving compliance with the new standard will be costly for many urban areas. Risk assessment efforts would benefit from delineating the biochemical mediators of ozone effects and from developing markers of exposure and ozone tissue dose for use in epidemiologic and controlled-exposure studies.

Individuals differ substantially in their responses to ozone, in terms of both airway function and inflammation, and these differences may be in part genetically determined (Torres et al. 1997). Lung function responses decline with age (Bascom et al. 1996). Smokers' airways are less responsive than the airways of nonsmokers (Frampton et al. 1997a), but smokers are nevertheless at risk of airway inflammation (Torres et al. 1997). No correlation has been found between the changes in pulmonary mechanics and airway inflammation following exposure to ozone (Torres et al. 1997; Balmes et al. 1996), suggesting that airway function effects and epithelial injury are mediated by different physiological processes. The biochemical processes that initiate these diverse effects have not been elucidated. Furthermore, no determinants or markers of susceptibility to ozone-related health effects have been identified.

Ozone is relatively insoluble in aqueous solutions but highly reactive, accounting for its ability to affect all airway generations. Ozone likely reacts completely within the epithelial lining fluid, and interacts with apical cell membranes only where epithelial lining fluid is markedly

attenuated (Pryor 1992). The protean effects of ozone exposure may thus be mediated in large part by reaction products of ozone.

Unsaturated fatty acids in surfactant and in cell membranes are predicted to be important targets for ozone reactivity (Uppu et al. 1995; Pryor et al. 1995). Reaction products of lipid ozonation may persist and penetrate cells to mediate some aspects of ozone toxicity. For example, various lipid ozonolysis products initiate signal transduction (Kafoury et al. 1998), are chemotactic for polymorphonuclear leukocytes (PMNs) (Madden et al. 1993), alter alveolar macrophage function (Hamilton et al. 1996), suppress T lymphocyte mitogenesis (Madden et al. 1993), and activate eicosanoid metabolism in airway epithelial cells (Leikauf et al. 1993). Products of the Criegee ozonation process include aldehydes (Pryor and Church 1991),

$$RCH{=}CHR' + O_3 + H_2O \rightarrow RCHO + R'CHO + H_2O_2,$$

which are sufficiently stable to be isolated and quantitated in epithelial lining fluid. Pryor and colleagues (1996) have proposed that aldehydes may serve as biomarkers of ozone reactivity with epithelial lining fluid lipids. Moreover, the relative yield of various aldehyde species should be predictable from the known fatty acid content of surfactant or membrane lipids (Uppu et al. 1995).

Aldehydes are identifiable in the BAL fluid of animals after ozone exposure. Pryor and colleagues (1996) found increases in hexanal, nonanal, and heptanal in BAL fluid from rats following 30- to 120-minute exposures to 2.5 ppm ozone. (This study is briefly reported in Appendix A.) Levels were highest immediately after exposure, declined substantially by 5 hours after exposure, and were back to baseline by 18 hours after exposure. Concentrations increased with the addition of CO_2 to the air, indicating that the increased ventilatory rate was accompanied by increased reaction of ozone with epithelial lining fluid lipids. This suggests that aldehyde concentrations in BAL fluid may reflect ozone tissue dose. Hamilton and colleagues (1996) have identified protein adducts of 4-hydroxynonenal, a product of lipid peroxidation, in BAL fluid from two human subjects exposed to ozone.

We hypothesized that exposure of healthy humans to ozone at concentrations found in ambient air causes both ozonation and peroxidation of lipids in epithelial lining fluid or epithelial cell membranes. Ozonation would yield nonanal from oleic acid and hexanal from any *n*-6 unsaturated fatty acid. In addition, hexanal can be produced from the ozone-initiated autoxidation of any *n*-6 polyunsaturated fatty acid. Both nonanal and hexanal would therefore be expected to increase in BAL fluid following ozone exposure. Because ozonation products may mediate diverse effects of ozone exposure, we also examined the relationship between nonanal and hexanal recovery from BAL fluid and ozone-induced changes in pulmonary function, airway inflammation, and epithelial permeability to proteins. Finally, these studies compared findings in healthy nonsmokers with those in current smokers, a population exposed daily to high burdens of inhaled oxidants.

METHODS AND STUDY DESIGN

This study was an outgrowth of an HEI-funded project, *Pulmonary Biomarkers for Ozone Exposure*, for which William A. Pryor served as principal investigator (see Appendix A), and was not part of the original plan for that project. The present study utilized BAL fluid collected from subjects in a previous investigation, for which Mark W. Frampton was the principal investigator, of the relationship between lung function responsiveness and airway inflammation following ozone exposure (*Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers*). The detailed methods and findings of that study have been reported (Torres et al. 1997; Frampton et al. 1997b). The pertinent aspects of study design and subject selection are described below, followed by the methods used in Dr. Pryor's laboratory to analyze the BAL samples for the current study.

EXPOSURE AND LAVAGE PROTOCOL

Subjects

Volunteers of both sexes aged 18 to 40 years were recruited from the local community through bulletin board and newspaper advertisements; most were college students. Informed consent was obtained, and the study was approved by the Research Subjects Review Board of the University of Rochester. Subjects were evaluated with a respiratory questionnaire modified from Lebowitz and colleagues (1975), physical examination, spirometry, and exercise testing on a bicycle ergometer. All were free of cardiorespiratory disease, denied symptoms of respiratory infection within the three weeks preceding exposure, completed the exercise protocol, and had normal spirometry without exercise-induced bronchoconstriction (the decrease in forced expiratory volume in one second [FEV_1] was less than 5% following 20 minutes of exercise at a minute ventilation [$\dot{V}_E$] of 25 L/min/m^2 body surface area). Nonsmokers had never used tobacco regularly, and had no tobacco use in the three years preceding the study. Smokers were currently smoking at least one pack per day, with at least three pack-years of smoking history.

Subjects were selected for this study based on lung function responsiveness after exposure to 0.22 ppm ozone for four hours, with intermittent exercise. This exposure is comparable to summertime outdoor exercise in cities such as Los Angeles or Mexico City. The methods for subject screening and the results of this classification exposure have been reported (Frampton et al. 1997a). For the purposes of this study, ozone "responders" and "nonresponders" were selected based on decrement (> 15%) or lack of decrement (< 5%) in FEV_1, respectively. Because of the low rate of ozone responsiveness among the smokers (Frampton et al. 1997a), smokers were considered as a single group. Thus three groups were studied: nonsmoker nonresponders ($n = 7$), nonsmoker responders ($n = 8$), and smokers ($n = 12$, including one ozone responder).

Study Design

Each subject underwent a total of three exposures and three BAL procedures, with each exposure-BAL sequence separated by at least three weeks. They were exposed once to air and twice to ozone, and BAL was performed immediately after one of the ozone exposures (referred to subsequently as "ozone early") and 18 hours after the other ozone exposure ("ozone late"). The BAL procedure was randomized to either early or late for each subject's air exposure. Volunteers were exposed in pairs, and were randomized so that one was scheduled to undergo BAL immediately after exposure and the other 18 hours after exposure. A restricted randomization scheme was employed to balance the various treatment assignments over time. Pairs of subjects were assigned to all three treatments (air early or late, ozone early, ozone late) using a single randomization.

All ozone exposures were 0.22 ppm ozone for four hours; exercise for 20 of each 30 minutes was sufficient to achieve a $\dot{V}_E$ of approximately 25 L/min/m^2 body surface area. The order of the exposures was randomized, and neither subjects nor investigators were informed of the exposure atmosphere. Smokers were not permitted to smoke during exposure, but were not advised to abstain from smoking prior to exposure.

Exposure Facilities

All exposures were undertaken in a 45-m^3 environmental chamber in the General Clinical Research Center at the University of Rochester, the characteristics of which have been described (Utell et al. 1984). Exercise bicycle ergometers and pulmonary function testing equipment are housed within the chamber, so that subjects were not required to exit the chamber for physiologic testing. For comfort, temperature and relative humidity were maintained at 21.2°C ± 0.92°C (mean ± SD) and 37.1% ± 3.0%, respectively.

All ozone exposures were conducted at a target concentration of 0.22 ppm (430 μg/m^3). Ozone generation was accomplished by passing Breathing Quality Oxygen through a flow meter into a water-cooled, high-voltage discharge ozonator (Model 03V5, Ozone Research and Equipment Corp., Phoenix, AZ). A portion of the ozonator output (1 L/min) was connected to the chamber air intake (10 m^3/min) through a Venturi mixer.

An ozone analyzer (Model 8810, Monitor Labs, Inc., Englewood, CO) continuously sampled the ozone concentration in the chamber atmosphere through a Teflon tubing connection. By means of feedback circuitry, the analyzer regulated the ozonator output. A Dasibi Ozone Analyzer (Model 1003-AH, Dasibi Environmental Corp., Glendale CA), designated as an EPA Transfer Standard, was used to calibrate the Monitor Labs analyzer. Before each ozone exposure, the calibration procedure required that the Monitor Labs analyzer be compared to the Transfer Standard while both instruments were sampling the identical ozone concentration produced by a portable ozone generator (Stable Ozone Generator, Model SOG-2, Ultraviolet Products Inc., San Gabriel, CA). The output of the portable ozone generator, in turn, was validated at least bimonthly against a certified ozone standard at the Air Quality Control Station of the NY State Department of Environmental Conservation (Avon, NY).

Air exposures, and the diluent air for the ozone exposures, used environmental air passed through an air intake purification system (Utell et al. 1984). The quality of the purified air as to background ozone, nitrogen oxides, and sulfur dioxide concentrations was established by the use of the Dasibi Model 8810 Ozone Analyzer, an NO_x Analyzer (Model 8840, Monitor Labs Inc., Englewood, CO), and a Meloy SO_2 Analyzer (Model SA285E, Columbia Scientific Instruments, Jollyville, TX), respectively. Background levels of air pollutants in the intake air of the chamber were at or below detection levels with respect to particles, sulfur dioxide, nitrogen oxides, and ozone: specifically, less than 4 μg particles/m^3, approximately 0.01 ppm NO_2, and less than 0.005 ppm for O_3 and sulfur dioxide.

Physiologic Testing

Spirometry was performed using a pneumotachograph interfaced with a computer (Microloop, Medical Graphics, St. Paul, MN). Forced vital capacity (FVC), FEV_1, and forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) were recorded from the best of three determinations, based on FEV_1. Airway resistance and thoracic gas volume were measured during panting using an

integrated-flow, pressure-corrected body plethysmograph. Specific airway conductance (*sG*aw) was determined as the reciprocal of airway resistance, corrected for thoracic gas volume. Minute ventilation was measured at rest and during exercise using inductive plethysmography (Respigraph Model PN SY01, NonInvasive Monitoring Systems, Miami Beach, FLA), calibrated with a rolling seal spirometer (Model 840, Ohio Medical Products, Houston, TX).

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed using fiberoptic bronchoscopy in both the lingula and the right middle lobe. Subjects were premedicated with 0.75 to 1.0 mg intravenous atropine, and topical anesthesia of the upper airway was established using lidocaine spray. Oxygen by nasal cannula was administered, and cardiac rhythm was monitored throughout the procedure. The fiberoptic bronchoscope (FB-19H, outer diameter 6.3 mm, Pentax, Orangeburg, NY) was passed orally, and topical lidocaine was administered through the bronchoscope to suppress cough. The bronchoscope was gently wedged in a subsegmental airway of the inferior segment of the lingula. Four 50-mL aliquots of sterile normal saline were sequentially instilled, immediately withdrawn under gentle suction, and collected into a siliconized Erlenmeyer flask on ice. The bronchoscope was then withdrawn and gently wedged in a subsegmental airway of the right middle lobe and the lavage was repeated.

Total and differential cell counts were performed as previously reported (Torres et al. 1997). Lavage fluids were then centrifuged to remove cells and the supernatant fluids were stored at –80°C until assayed. Fluids used for analysis of aldehydes were from BAL of the right middle lobe in all subjects.

Measurement of Proteins in Bronchoalveolar Lavage Fluid

Concentrations of total protein, albumin, and IgM were determined to provide indices of changes in epithelial permeability. Bronchoalveolar lavage fluids were stored at –80°C prior to analysis; determinations were done simultaneously on all samples from each subject. Immunoassays were validated for BAL fluid using serial dilutions and "add back" of purified antigen to confirm accurate recovery. Total protein was determined using the method of Lowry and colleagues (1951), with crystalline bovine serum albumin as the standard. Albumin was measured using a modified antibody-capture enzyme-linked immunosorbent assay (ELISA) as described previously (Frampton et al. 1989). IgM was measured using a sandwich ELISA with sensitivity in the range of 5 to 200 ng/mL. Human IgM (standard), murine anti–human IgM (primary antibody), peroxidase-conjugated goat anti–human IgM (secondary antibody), and orthophenylene-diamine-dihydrochloride (color indicator) were obtained from Sigma Chemicals (St. Louis, MO).

ALDEHYDE ANALYSIS PROTOCOL

The chemicals not otherwise specified below were purchased from Sigma Chemical Co. (St. Louis, MO). As analytical standards, nonanal was purchased from Alfa Products (Danvers, MA; purity 98%) or from Aldrich (Milwaukee, WI; purity 95%); hexanal was purchased from Aldrich (purity 98%). These standards were further purified by passage through a silica solid-phase extraction cartridge (Supelco, Bellefonte, PA), and then sealed under nitrogen, as previously described for hexanal and propanal (Frankel et al. 1989).

The aldehydes were analyzed as oximes of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) by gas chromatography (GC) using electron capture detection (ECD). This method was designed by Glaze and colleagues (1989) for the analysis of aldehydes produced in the treatment of drinking water and was modified and improved by Cueto and associates (1992, 1994) for application to lavage fluid.

Briefly, 2 mL of a solution containing hexanal or nonanal (1 to 20 µg/L) or 5 mL of BAL fluid was allowed to react with 0.5 mL of a PFBHA solution (1.0 mg/mL) for two hours. Then 3 drops of 18N H_2SO_4 was added and the oximes were extracted with 1 mL of hexane containing decafluorobiphenyl (50 µg/L) as the internal standard. The hexane layer was then washed with 5 mL of 0.1N H_2SO_4 and dried over anhydrous sodium sulfate. A Hewlett Packard Model 5890 series II gas chromatograph with a ^{63}Ni electron capture detector and an autosampler (Hewlett Packard 7361A), connected to a cool on-column injector with electronic pressure control, was used for the analysis. An HP-5 25-m × 0.2-mm × 0.33-µm column with a 5-m × 0.53-mm retention gap was used for the separation. Helium (0.9 mL/min) was used as a carrier, and argon-methane was used as a makeup gas. The chromatographic conditions were as follows: detector temperature, 280°C; temperature programming, 50°C for 1 minute; temperature ramp, 5°C/min; final temperature, 220°C. Two microliters of sample was injected. The area of the chromatograph peak was divided by the area of the internal standard peak (decafluorobiphenyl) and expressed as nanomoles of aldehyde per liter of BAL fluid, based on standard calibration curves.

DATA HANDLING AND STATISTICAL METHODS

The primary analyses for the data from BAL were based on a two-way mixed model or repeated-measures analysis of variance (ANOVA), which included one between-subjects factor and two within-subject factors. The between-subjects effect was subject group. The primary within-subject effect in the model was treatment: air, ozone early, or ozone late (three conditions). For these analyses the air exposure was considered as a single treatment, regardless of whether subjects underwent lavage early or late. This assumption was checked in a separate two-way ANOVA comparing air early with air late; no significant differences were found.

The second within-subject factor was a period effect. Because subjects were studied at different times of the year, period effects were not expected, and the effect was included as a check of this assumption. As in any repeated-measures ANOVA, a random subject effect was also included, which was nested within groups. The analysis also included a test of interaction between the treatment effect and the subject group. If this interaction was significant, then we concluded that differences among the three treatments were different among the three groups of subjects. If the interaction was not significant, then the individual or main effects were examined for statistical significance. No terms were included in the model for carry-over effects because of the relatively long interval between repeated measurements on the same subject.

Each analysis included an examination of residuals as a check on the assumptions of normally distributed errors with constant variance. In many instances the residual analysis indicated that the variance was not constant. In these cases a log transformation proved effective in stabilizing the variance.

Table 1. Percentage of Change in FEV_1 of Exercising Subjects Immediately After Exposure to 0.22 ppm Ozone or Air for 4 Hours[a]

Exposure Group	Air	Ozone Early	Ozone Late
Smoker (n = 12)	+5.0 ± 1.0	−1.0 ± 2.3	−2.5 ± 2.8
Nonsmoker responder (n = 7)	+6.0 ± 1.4	−29.1 ± 3.7	−31.1 ± 3.3
Nonsmoker nonresponder (n = 8)	+3.0 ± 1.0	−2.7 ± 2.2	−5.1 ± 1.8

[a] Lung function was measured immediately after each exposure. Data are expressed as means ± SE.

For a small number of endpoints, analysis of covariance (ANCOVA) was performed to examine predictors of the changes in aldehyde levels. The dependent variables for these analyses were differences in hexanal and nonanal between ozone early and air exposure. Covariates included age, sex, change in PMN concentration, and estimated intake of ozone. A residual analysis was included and outliers were removed for these analyses.

A level of 5% was required for statistical significance.

RESULTS

Achieved ozone concentration (mean ± SD) for the exposures in this study was 0.219 ± 0.004 ppm. Ambient outdoor ozone levels during the study were obtained from the Department of Environmental Conservation of New York State, and did not exceed 0.10 ppm within the 24 hours prior to any exposure session. The three subject groups did not differ with regard to age, baseline FEV_1, or $\dot{V}_E$ during exposure.

Pulmonary function responses to these exposures have been reported (Frampton et al. 1997a,b). In brief, decrements in FEV_1 (Table 1) and FVC immediately following each ozone exposure were similar to those following the initial classification ozone exposure, which demonstrates that subjects remained responders or nonresponders to ozone as originally classified. For ozone responders, FEV_1 returned to near baseline at 18 hours after exposure (data not shown). Table 1 shows percentage of change in FEV_1 for subject groups.

AIRWAY INFLAMMATION AND INJURY

The airway inflammatory response to ozone in this study has been reported previously (Torres et al. 1997; Frampton et al. 1997b). All three subject groups showed evidence of airway inflammation in response to ozone exposure in that increased numbers of PMNs and lymphocytes were recovered by BAL after ozone exposure compared with those recovered after air exposure (Figure 1). The influx of PMN was greater 18 hours after exposure than immediately after exposure in all groups, and no significant difference between groups was found in the intensity or time course of the response.

Protein influx into BAL fluid was measured as an index of changes in epithelial permeability following ozone exposure. In addition to total protein, albumin and IgM were selected as molecules of divergent size whose source is from plasma exclusively. As shown in Figure 2, total protein, albumin, and IgM increased in response to ozone exposure. Analysis of variance revealed no significant differences among groups for the increases in total protein, but

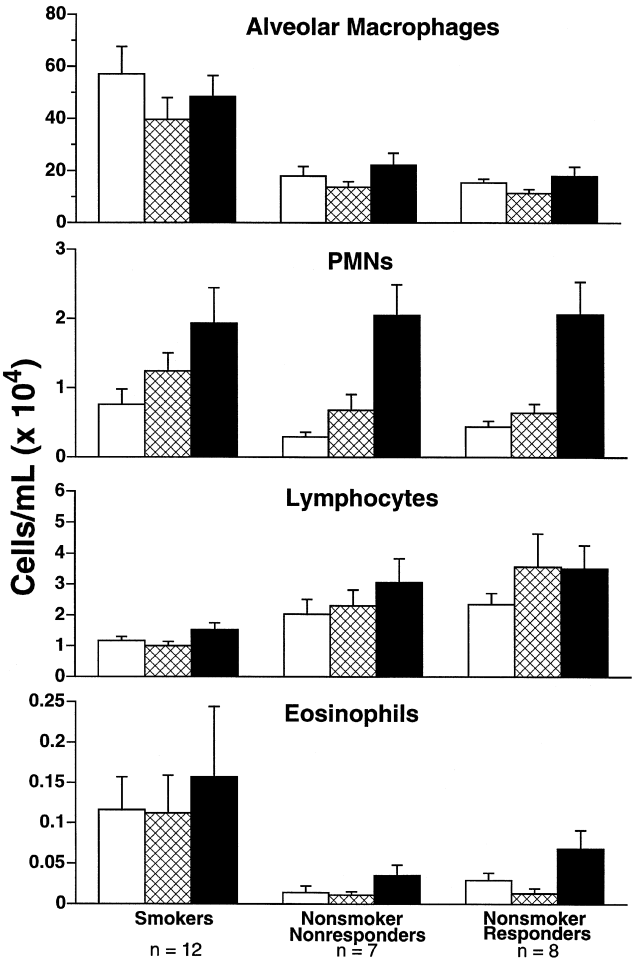

Figure 1. Cell recovery from BAL fluid. Alveolar macrophages were more numerous in smokers than nonsmokers (group effect, $p < 0.0001$), and decreased early after ozone exposure in all groups (ozone effect, $p < 0.0001$). PMNs progressively increased after ozone exposure for all groups (ozone effect, $p < 0.0001$), and were increased in smokers compared with nonsmokers after air and early after ozone (interaction between group and ozone effects, $p = 0.011$). Lymphocytes were decreased in smokers compared with nonsmokers (group effect, $p < 0.0001$), and increased late after ozone in all groups (ozone effect, $p < 0.0001$). Eosinophils showed no significant ozone effect. Data are expressed as means ± SE. Open bars: air exposure; cross-hatched bars: ozone early (BAL immediately after ozone exposure); solid bars: ozone late (BAL 18 hours after ozone exposure).

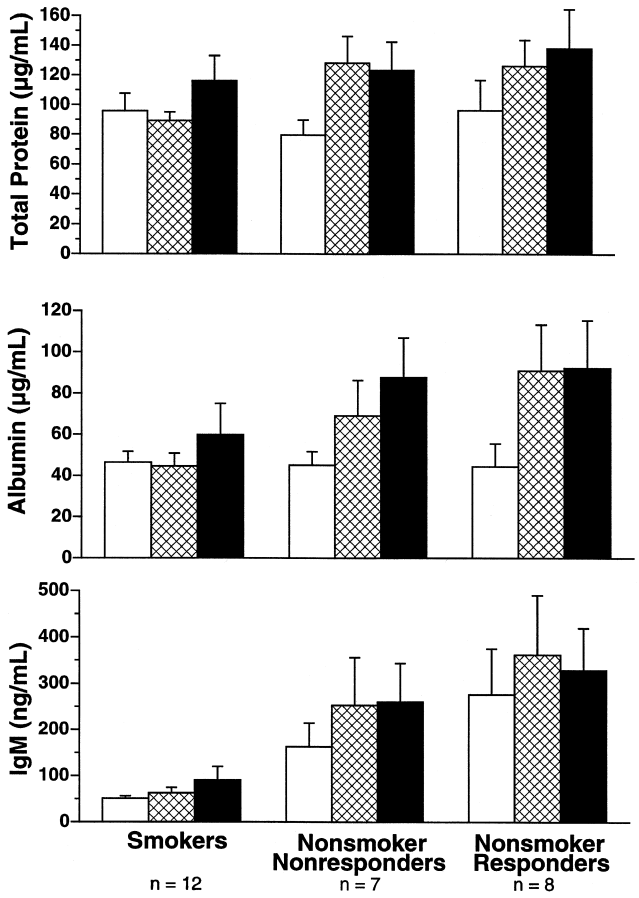

Figure 2. Concentration of total protein, albumin, and IgM in BAL fluid. Total protein, albumin, and IgM increased after ozone exposure ($p < 0.003$ for all). The increase in albumin was delayed in smokers compared with nonsmokers (ANOVA interaction between group and ozone effect, $p = 0.033$). Data are expressed as means ± SE. Open bars: air exposure; cross-hatched bars: ozone early (BAL immediately after ozone exposure); solid bars: ozone late (BAL 18 hours after ozone exposure).

a highly significant effect of ozone exposure ($p < 0.0001$). The increase in albumin following ozone exposure was delayed for smokers compared with nonsmokers. Albumin showed the greatest increase and IgM the least, consistent with a permeability effect.

ALDEHYDES

Results of quantitation of hexanal and nonanal in BAL fluid are shown in Table 2 and Figure 3; complete results of the ANOVA are included in Tables 3 and 4. Both hexanal and nonanal were detectable in all samples measured. Ozone exposure resulted in a significant early increase in nonanal in all groups (ANOVA treatment effect, $p < 0.0001$), with no significant difference among groups. Although the mean hexanal level increased after ozone exposure in all groups, the difference was not statistically significant ($p = 0.16$). Both nonanal and hexanal levels returned to baseline by 18 hours after exposure. Lavage fluid from smokers contained less hexanal than that from nonsmokers after both air and ozone exposure (group effect, $p = 0.049$). The levels of hexanal and nonanal after air exposure were similar when BAL was done either early or late (data not shown), indicating that timing of BAL did not influence the findings.

Analysis of covariance revealed no significant relationship between aldehyde levels and group, confirming the absence of a relationship between aldehyde levels and changes in pulmonary mechanics following ozone

Table 2. Aldehyde Concentrations in Bronchoalveolar Lavage Fluid After Exposure to Air or Ozone[a]

Exposure Group	Hexanal			Nonanal		
	Air	Ozone Early	Ozone Late	Air	Ozone Early[b]	Ozone Late
Smoker (n = 12)	1.76 ± 0.59	3.70 ± 1.17	1.40 ± 0.36	4.16 ± 0.77	5.00 ± 0.78	2.55 ± 0.40
Nonsmoker responder (n = 7)	4.46 ± 1.46	8.55 ± 2.52	5.24 ± 1.11	2.89 ± 0.77	5.88 ± 1.05	4.51 ± 1.46
Nonsmoker nonresponder (n = 8)	4.25 ± 1.65	5.79 ± 2.69	3.55 ± 1.30	3.23 ± 0.49	6.04 ± 0.94	2.66 ± 0.34

[a] Data are nanomoles of aldehyde per liter of BAL fluid, and are expressed as means ± SE.

[b] $p < 0.0001$ for ozone effect by ANOVA.

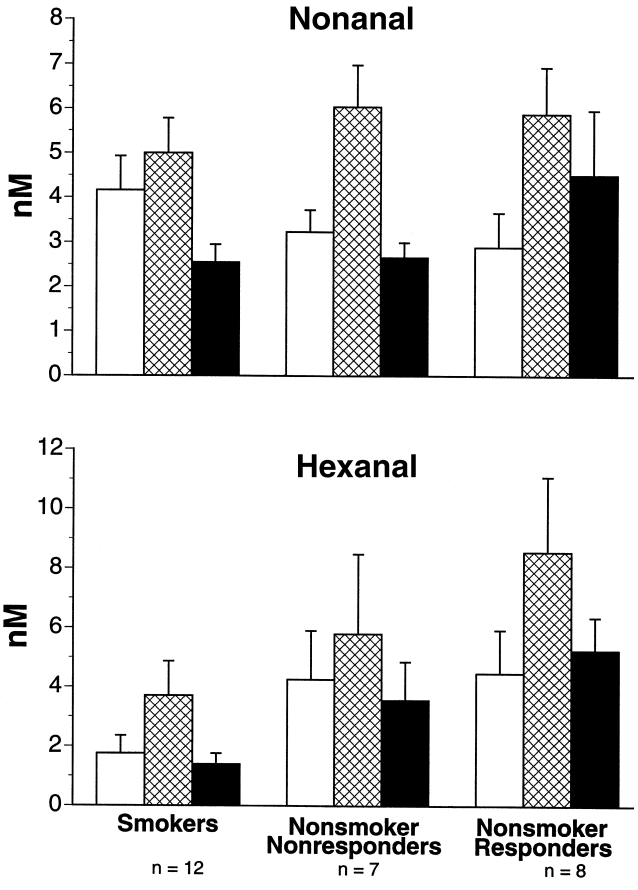

Figure 3. Recovery of nonanal and hexanal in BAL fluid. Data are nanomoles of aldehyde per liter of BAL fluid, and are expressed as means ± SE. Open bars: air exposure; cross-hatched bars: ozone early (BAL immediately after ozone exposure); solid bars: ozone late (BAL 18 hours after ozone exposure).

exposure (Tables 3 and 4). There was also no relationship between aldehyde levels and changes in PMNs, calculated estimated ozone dose, subject age, or BAL levels of total protein or albumin. The increases in nonanal correlated with the increases in hexanal early after ozone exposure ($r = 0.55$, $p = 0.034$).

DISCUSSION

These studies demonstrate that aldehydes are detectable in BAL fluid from human smokers and nonsmokers, and that the level of nonanal increases following four-hour exposures to 0.22 ppm ozone with exercise. Levels of hexanal also increased, but not significantly as evaluated by ANOVA. This may be related to limited statistical power associated with the relatively small number of subjects. Levels of both nonanal and hexanal returned to baseline by 18 hours after exposure. These findings suggest that exposure to ozone at environmentally relevant levels, with exercise, results in production of lipid ozonation products in the human lung.

Recovery of hexanal in BAL fluid was less in smokers than in nonsmokers (Figure 3). Explanations for this may include alterations in lipid composition of epithelial lining fluid of smokers, increased levels of antioxidants in epithelial fluid, or increased epithelial permeability to plasma proteins that form adducts with aldehydes in BAL fluid (Hamilton et al. 1996). However, the pattern of response to ozone for smokers was similar to that for nonsmokers for both hexanal and nonanal in spite of the smokers' significant daily exposure to oxidants (Morrow et al. 1995) and the thicker mucous layer covering the airways associated with smoking (Fiore et al. 1994). This surprising finding suggests that airways already exposed to a

Table 3. Log-Transformed Analyses of Variance for Nonanal

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*	*p* Value
Treatment	2	6.50	3.25	12.1	< 0.0001
Order	2	1.68	0.84	3.12	0.054
Group	2	0.34	0.17	0.23	0.80[a]
Subject (Group)	24	17.61	0.73	2.72	0.0019
Group × Treatment	4	1.64	0.41	1.52	0.21
Error	44	11.85	0.27		
Total	78	39.08			

[a] The error term for the Group effect was the Subject (Group) mean square coefficient of determination (R^2) = 69.7%.

Table 4. Log-Transformed Analyses of Variance for Hexanal

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*	*p* Value
Treatment	2	3.06	1.53	1.89	0.16
Order	2	0.51	0.25	0.31	0.73
Group	2	28.71	14.36	3.42	0.049[a]
Subject (Group)	24	100.6	4.19	5.16	< 0.0001
Group × Treatment	4	0.75	0.19	0.23	0.92
Error	43	34.91	0.81		
Total	77	174.6			

[a] The error term for the Group effect was the Subject (Group) mean square coefficient of determination (R^2) = 80.0%.

substantial oxidant burden remain susceptible to ozone-induced lipid oxidation. Furthermore, this observation is consistent with the finding that airway inflammation following ozone exposure is similar for smokers and nonsmokers, despite differences in the airways' functional responsiveness to ozone (Torres et al. 1997; Frampton et al. 1997a).

We found no relationship between hexanal or nonanal levels and other ozone-related effects, including pulmonary function responses, airway inflammation, or increases in total protein or albumin. We also observed no relationship between aldehyde increases and subjects' sex or age. Aldehyde levels did not correlate with estimated ozone dose, although the use of only one exposure concentration in this study does not provide sufficient data for evaluating the concentration-response relationship. Larger studies with a range of ozone exposure levels will be required to determine whether aldehydes may serve as markers of airway injury, inflammation, or other effects. However, this study establishes that hexanal and nonanal levels are unrelated to lung function responsiveness after exposure to ozone. Furthermore, because respiratory symptoms occur more often in ozone responders (Frampton et al. 1994), increases in nonanal are not linked with increased symptoms. These findings suggest that reactive aldehydes do not play a significant role in the airway irritant receptor response to ozone.

The current data show both similarities and differences to experiments in rats exposed to ozone. Pryor and colleagues (1996; see also Appendix A) measured hexanal, heptanal, and nonanal levels in BAL fluid from rats exposed to ozone at various concentrations and durations, with and without 5% CO_2 to increase ventilation. Both hexanal and nonanal increased following ozone exposure, with levels dependent on ozone concentration and exposure duration. Levels were increased by inhalation of 5% CO_2, simulating the effects of exercise. The lowest concentration of ozone used in the rat studies, 0.5 ppm, caused a gradual increase in aldehydes over 90 minutes of exposure, from approximately 10 to 80 nM, and then fell off slightly at 120 minutes. Levels returned to baseline by 18 hours after exposure.

In the human studies, baseline and postexposure concentrations of hexanal and nonanal were lower than in the rat studies; however, this may be related to differences in dilution of epithelial lining fluid because of the lavage procedure. Rat epithelial lining fluid volume, including both airway and alveolar compartments, has been esti-

mated at approximately 0.08 mL (Hatch 1991). In the rat studies, 12 mL of saline was instilled and reinstilled a total of three times, resulting in a 150-fold dilution (12/0.08). Human epithelial lining fluid volume of the alveolar space has been estimated at 8.9 mL (Hatch 1991). Because approximately 1% of the human lung is sampled during BAL (Linder and Rennard 1988), the epithelial lining fluid volume sampled can be estimated to be 0.09 mL. The human BAL procedure utilized a total of 200 mL of saline, giving a dilution factor of 200/0.09 = 2222, or approximately 15-fold greater dilution in the human studies than in the rat studies. If we correct for epithelial lining fluid dilution and use values for rat nonanal levels from Figure 2 of Pryor and colleagues (1996), the nonanal concentration in rat epithelial lining fluid at baseline was approximately 1.5 μM, and for nonsmoking humans was 6.7 μM. Concentrations of nonanal increased approximately eightfold in the rat after 90 minutes of exposure to 0.5 ppm with 5% CO_2, and in nonsmoking humans it increased twofold following exposure to 0.22 ppm ozone for four hours with intermittent exercise. Levels returned to baseline by 18 hours after exposure in both the human and rat studies. Thus baseline and postexposure concentrations of nonanal in epithelial lining fluid appear to be of the same order of magnitude in rats and humans.

Both nonanal and hexanal increased to a similar degree in rats following ozone exposure, whereas in humans the increase in hexanal was not significant. It is unclear whether the smaller increase in hexanal in humans compared with rats represents a species difference or the effect of a lower exposure concentration. It may depend on the fact that hexanal, unlike nonanal, arises from both Criegee ozonation and from ozone-initiated lipid peroxidation. The relative importance of these processes may differ between species.

In conclusion, these studies confirm that exposure to ozone with exercise, at concentrations relevant to urban outdoor air, results in ozonation of lipids in epithelial lining fluid and generation of nonanal. This effect occurs independently of smoking status or decrements in lung function following exposure. Furthermore, in this study, nonanal levels did not correlate with indices of airway inflammation or injury. Further studies are needed to evaluate the utility of nonanal as a marker or dosimeter of ozone exposure, and to determine the role of reactive aldehydes in the airway effects of ozone.

ACKNOWLEDGMENTS

The research group in Rochester acknowledges the valuable technical assistance of Mitra Azadniv, David Chalupa, Lauren Frasier, F. Raymond Gibb, and Donna Speers. Work at the Biodynamics Institute in Louisiana was performed by Drs. G.L. Squadrito, M.G. Salgo, E. Bermúdez, and R.M. Uppu, whose contributions are greatly appreciated.

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APPENDIX A. Detection of Aldehydes in Bronchoalveolar Lavage Fluid from Rats Exposed to Ozone

This Appendix[†] describes the methods and results related to Specific Aim 1 of Dr. William A. Pryor's HEI Project Report, *Pulmonary Biomarkers for Ozone Exposure* (Pryor et al. 1999 is available on request from HEI). As part of this Specific Aim, Pryor and colleagues identified the aldehydes present in BAL fluid of rats exposed to various concentrations of ozone, with and without CO_2 to increase the rats' breathing rate and mimic human exercise conditions (see also Pryor et al. 1996).

The other specific aims in Dr. Pryor's Project Report included a study of the biological effects of lipid ozonation products, and the use of chemical and electron spin resonance to identify the mechanism of free radical production from the interaction between ozone and pulmonary target molecules.

The results included in this Appendix serve to aid the reader in comparing the aldehydes found in the BAL fluid from ozone-exposed rats with the results of Frampton and Pryor's study of exercising humans, which are presented in the main portion of this Investigators' Report.

EXPERIMENTAL METHODS

Animal Exposures

Ninety-day-old, male, Sprague-Dawley, specific pathogen–free rats weighing 300 to 330 g (Harlan Sprague-Dawley, Houston, TX) were used. Rats were acclimatized in standard cages with access to food (Harlan Teklad, Madison, WI) and water for at least five days prior to exposure. Twelve-hour day-night lighting intervals were maintained. Rats were randomized into control and experimental groups. The animals were weighed and then transferred to stainless-steel open-mesh cages (one per cage) and placed in the exposure chamber as described by Hinners and colleagues (1968). The rats were exposed to filtered air, filtered air plus 5% CO_2, ozone, or ozone plus 5% CO_2. Ozone concentrations of 0.5, 1.2, 2.5, 5.0, and 10.0 ppm were used and exposure times of 30, 60, 90, 120, and 240 minutes. Animals had access to water but not food during the exposure.

The chamber was a 0.25-m^3 whole-body exposure chamber (Air Dynamics, Inc., Baton Rouge, LA). The air flow rate was adjusted to give 12 chamber volume changes per hour. Ozone was generated by passing compressed air (1.0 L/min) through a Sander Ozonizer (Model 200, Sander Aquarientechnik, AM Osterberg, Germany) and then diluting it with filtered room air to the desired concentration. The concentration of ozone in the exposure chamber was monitored continuously via a probe in the geometrical center of the chamber using an ozone analyzer (Dasibi model 1008-AH, Dasibi Environmental Corp., Glendale, CA) connected to a strip-chart recorder. The ozone analyzer was calibrated using a calibration kit (Enmet Analytical, Ann Arbor, MI). The chamber also was tested for the homogeneity of the distribution of ozone concentrations.

Carbon dioxide was produced from a pressurized cylinder of CO_2 and diluted to the desired concentration with filtered air; the flow of CO_2 into the chamber was 0.5 L/min. The CO_2 concentration was continuously monitored using a Beckman medical gas analyzer (Model LB-2, Beckman Instruments, Fullerton, CA) connected to a strip-chart recorder. The CO_2 analyzer was calibrated using a standard of 1.2% CO_2 in nitrogen (Aldrich, Milwaukee, WI).

All the procedures used in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee.

Exposure Conditions

The final (mean ± SD) ozone exposure concentrations were as follows: 0.5 ± 0.1, 1.2 ± 0.2, 2.5 ± 0.2, 5.0 ± 0.5, and 10.0 ± 0.9 ppm. The average relative humidity and temperature were 58.3% ± 7.2% and 21° ± 1°C, respectively. Carbon dioxide concentration (5.0% ± 0.2%) was stable throughout the entire exposure time.

Bronchoalveolar Lavage Technique

Upon termination of the exposure, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The rats were killed by exsanguination and a thoracotomy was performed to expose the lungs and the trachea; a cannula was inserted into the trachea and secured using a silk suture. Then 12 mL of phosphate-buffered saline (0.9% NaCl:50 mM sodium phosphate, pH 7.4; 37°C) was injected into the lungs via a syringe and the tracheal cannula. The infusion was allowed to remain for 30 seconds, and then was retrieved and reinstilled for a total of three washes with the same solution. An average of 9.0 ± 2.0 mL BAL fluid was recovered from each rat. Lavage fluid was stored at –70°C immediately after it was obtained and was used for detecting ozonation products one, two, or three weeks later; no differences due to length of storage or to centrifuging the BAL fluid and removing the cells were observed (data not shown). For

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derivatization and analysis, 5.0 mL of the BAL fluid was used (as described in the Aldehyde Analysis Protocol section of the main report) and the results were expressed as the concentration of a given aldehyde in the BAL fluid.

To study the in vivo lifetime of aldehydes after exposure, 12 rats were exposed to 2.5 ppm ozone plus 5% CO_2 for 60 minutes. Immediately after exposure, 3 of the 12 rats were killed and subjected to the lavage procedure within 20 to 60 minutes. The remaining rats were allowed to recover for 5, 18, or 24 hours (3 rats per group) and then were killed. Lavage fluid was then collected from each rat as described above.

Aldehyde Analyses

The detectability limits for aldehydes can be greatly increased by derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) (Glaze et al. 1989; Cueto et al. 1994), a reagent that converts aldehydes to oximes containing five fluorine atoms (a pentafluorobenzyl group) that are particularly sensitive analytes for GC analysis with ECD. The sensitivity using the PFBHA method with capillary GC-ECD is in the picogram range. This method requires a rather labor-intensive derivatization and extraction procedure, but its sensitivity and ability to separate a very large number of aldehydic products makes it the method of choice for in vivo studies. This method has proven to be very powerful and extremely selective and sensitive (Cancilla and Que Hee 1992).

The aldehydes were analyzed as oximes of PFBHA by GC-ECD. These methods are described in detail in the main report.

Gas Chromatography with Electron Capture Detection Analysis

The analysis was conducted on a Hewlett Packard 5890 series II GC with a ^{63}Ni electron capture detector provided with a Hewlett Packard 7361A autosampler connected to a cool on-column injector with electronic pressure control. An HP-5 30-m × 0.53-mm × 2.65-µm (or 10-m × 0.53-mm × 2.65-µm) column with a 1-m × 0.53-mm retention gap was used for the separation. Helium (8 mL/min) was used as a carrier gas, and argon-methane as a makeup gas. The chromatographic conditions were: injector temperature, 250°C; detector temperature, 280°C; temperature programming, 50°C isothermal for 1 minute, 5°C/min to 220°C; split ratio, 20:1. One microliter of sample was injected.

RESULTS AND DISCUSSION

In pilot experiments, it appeared that the sensitivity of our method for detecting aldehydes would be a limiting factor, and we elected to use 5% CO_2 with ozone in order to increase the tidal volume of the exposed rats. The data in Table A.1 for exposure times of 30 to 90 minutes show that hexanal, heptanal, and nonanal are all produced in higher yields if 5% CO_2 is incorporated in the experimental design (see also Pryor et al. 1996). Therefore, experiments were done using 5% CO_2 at 0.5 ppm, 1.2 ppm, and 2.5 ppm ozone over a number of time points up to 4 hours. Some of

Table A.1. Aldehydes Measured in the Lavage Fluid of Rats Exposed to 2.5 ppm Ozone, 2.5 ppm Ozone + 5% Carbon Dioxide, Filtered Air, or Filtered Air + 5% Carbon Dioxide[a,b]

Exposure Time (minutes)	Hexanal (nM)		Heptanal (nM)		Nonanal (nM)	
	2.5 ppm O_3	2.5 ppm O_3 + 5% CO_2	2.5 ppm O_3	2.5 ppm O_3 + 5% CO_2	2.5 ppm O_3	2.5 ppm O_3 + 5% CO_2
30	65 ± 7	147 ± 40	27 ± 3	64 ± 13	69 ± 4	112 ± 27
60	55 ± 15	184 ± 24	34 ± 48	88 ± 16	73 ± 24	131 ± 18
90	72 ± 32	111 ± 28	52 ± 39	54 ± 20	68 ± 46	78 ± 6
120	63 ± 30	65 ± 26	29 ± 15	17 ± 6	62 ± 24	46 ± 26
240	20 ± 10	11 ± 4	33 ± 10	24 ± 6	11 ± 7	9 ± 7
	Air	Air + 5% CO_2	Air	Air + 5% CO_2	Air	Air + 5% CO_2
Pooled[c]	8 ± 3	12 ± 1	4 ± 1	7 ± 4	7 ± 2	13 ± 2

[a] Values are presented as means ± SD.

[b] n = 6 for each ozone exposure group.

[c] Results are pooled averages for 30- and 60-minute control groups for each exposure combination; values comparing air alone and air + CO_2 for each aldehyde did not differ significantly. Pooled n = 14.

Table A.2. Aldehydes Measured in the Lavage of Rats Exposed to 0.5 ppm Ozone + 5% Carbon Dioxide, 1.2 ppm Ozone + 5% Carbon Dioxide, or Filtered Air + 5% Carbon Dioxide[a,b]

Exposure Time (minutes)	Hexanal (nM)		Heptanal (nM)		Nonanal (nM)	
	0.5 ppm O_3 + 5% CO_2	1.2 ppm O_3 + 5% CO_2	0.5 ppm O_3 + 5% CO_2	1.2 ppm O_3 + 5% CO_2	0.5 ppm O_3 + 5% CO_2	1.2 ppm O_3 + 5% CO_2
30	59 ± 15	20 ± 5	15 ± 8	11 ± 4	48 ± 21	32 ± 14
60	50 ± 11	35 ± 5	11 ± 3	14 ± 2	35 ± 9	40 ± 5
90	84 ± 26	45 ± 23	27 ± 15	15 ± 8	72 ± 38	35 ± 20
120	33 ± 20	56 ± 24	7 ± 4	23 ± 13	39 ± 20	58 ± 34
	Air	Air + 5% CO_2	Air	Air + 5% CO_2	Air	Air + 5% CO_2
Pooled[c]	12 ± 1		7 ± 4		13 ± 2	

[a] Values are presented as means ± SD.

[b] n = 6 for each ozone exposure group.

[c] Results show the pooled averages for control groups exposed for 30, 60, 90, and 120 minutes. Pooled n = 14.

these data are shown in Table A.1 and some in Table A.2 (see also Pryor et al. 1996).

The autoxidation of *n*-6 polyunsaturated fatty acid can produce hexanal, but monounsaturated fatty acids would not be expected to undergo autoxidation (Cueto et al. 1994). Thus, we hoped aldehydes derived from monounsaturated fatty acids, such as oleic and palmitoleic acids, would be relatively specific biomarkers for exposure to ozone (Cueto et al. 1994). Of these two, oleic acid is more prevalent in the lungs of both rats and humans. Therefore, we studied the apparent yields of nonanal over a wide range of ozone concentrations, as shown in Table A.3.

Using 2.5 ppm ozone plus 5% CO_2 for 60 minutes, an exposure that gave a high yield of aldehydes, we followed the decay of the aldehyde signal with time after the end of the exposure period. As shown in Table A.4, all three of the aldehydes could be detected 5 hours after the end of the exposure, but at 18 and 24 hours, only hexanal remained elevated, undoubtedly because of continuing autoxidation in the rat lung following the exposure to ozone.

Table A.3. Nonanal Measured in the Lavage Fluid of Rats Exposed to Different Concentrations of Ozone for 60 Minutes

Exposure Group[a]	Nonanal[b] (nM)
Filtered air[c]	7.3 ± 1.7
Filtered air + 5% CO_2 [c]	13.4 ± 2.2
0.5 ppm O_3 + 5% CO_2	34.9 ± 8.8
1.2 ppm O_3 + 5% CO_2	39.8 ± 4.6
2.5 ppm O_3	73.0 ± 24.4
2.5 ppm O_3 + 5% CO_2	131.3 ± 6.1
5.0 ppm O_3	36.8 ± 13.4
5.0 ppm O_3 + 5% CO_2	102.2 ± 2.7
10.0 ppm O_3[d]	50.3 ± 22.9

[a] n = 6 rats for each exposure group, unless otherwise indicated.

[b] Values are presented as means ± SD.

[c] n = 14.

[d] n = 4.

Table A.4. Aldehydes Measured in the Lavage Fluid of Rats Exposed to 2.5 ppm Ozone + 5% Carbon Dioxide for 60 Minutes and Allowed to Recover for 0, 5, 18, or 25 Hours[a]

Timing of BAL After Exposure[b]	Hexanal (nM)	Heptanal (nM)	Nonanal (nM)
Immediately[c]	184.1 ± 24.0	87.8 ± 15.7	131.3 ±17.6
5 Hours	41.0 ± 29.3	8.2 ± 14.0	21.2 ± 9.3
18 Hours	5.0 ± 2.3	0	0
24 Hours	4.3 ± 0.3	0	0

[a] Values are presented as means ± SD.

[b] n = 3 for each group.

[c] "Immediately" means that BAL was performed as soon as possible, using the procedure described in the Bronchoalveolar Lavage Technique section; this process took 20 to 60 minutes and no differences were observed in the amount of BAL fluid recovered.

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OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS AND OTHER TERMS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
ECD	electron capture detection
ELISA	enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
$FEF_{25–75}$	forced expiratory flow between 25% and 75% of FVC
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GC	gas chromatography
H_2SO_4	sulfuric acid
IgM	immunoglobulin M
MS	mass spectrometry
ozone early	BAL performed immediately after ozone exposure
ozone late	BAL performed 18 hours after ozone exposure
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride
PMNs	polymorphonuclear leukocytes
*sG*aw	specific airway conductance
$\dot{V}_E$	volume of expired air in one minute (minute ventilation)

CRITIQUE
Health Review Committee

INTRODUCTION

Ozone, a highly reactive gas, is a major constituent of smog and an airway irritant. Some people experience transient declines in lung function and respiratory tract inflammation after exposure to ozone (Balmes et al. 1996; U.S. Environmental Protection Agency 1996). Studies with laboratory animals have demonstrated pathologic and physiologic effects of ozone on the respiratory system that are dependent on the dose and duration of exposure. Ozone's high reactivity make it unlikely to penetrate far beyond the fluid that lines the lung's epithelial cell layer. Its harmful effects are thought to be mediated by products of its reactions with constituents of the lining fluid and the epithelial cell membrane (Pryor 1992). These products include aldehydes (Pryor and Church 1991) which, although rapidly metabolized, can be toxic to cells (Leikauf et al. 1995).

Some inhaled pollutants react with intracellular molecules to form quantifiable products that reflect the dose received by a tissue; such products are considered to be biomarkers of dose. However, no biomarker for ozone has been identified. Methods to determine the dose of ozone to tissues would aid researchers in extrapolating the results of animal studies to humans.

In 1990, HEI issued RFA 90-3, "Ozone Interactions with Biological Macromolecules and Molecular Dosimetry." Under this RFA, HEI supported Dr. William A. Pryor, of Louisiana State University, to develop methods for measuring ozone reaction products and to determine if aldehydes derived from such reactions could serve as pulmonary biomarkers of ozone exposure.* During the study period, Dr. Mark Frampton, of the University of Rochester, provided Pryor with samples of epithelial cell lining fluid obtained by bronchoalveolar lavage (BAL)† during an earlier HEI-funded study of humans exposed under controlled conditions to filtered air or air containing ozone (Frampton et al. 1997). In the pilot study reported here, Pryor and colleagues analyzed these samples for the aldehydes hexanal and nonanal, which are formed by ozone's oxidation of unsaturated fatty acids. (Hexanal is formed by oxidation of a six-carbon unsaturated fatty acid found in lipids in epithelial lining fluid or epithelial cell membranes. Nonanal is formed by oxidation of a nine-carbon unsaturated fatty acid found in the same sources.) The Investigators' Report presents the results of this collaborative pilot study§, which was evaluated by the HEI Health Review Committee. This Critique is intended to aid the sponsors of HEI and the public by highlighting the strengths and limitations of the study and by placing the Investigators' Report into scientific perspective.

STUDY GOAL AND DESIGN

The goal of this pilot study was to determine whether aldehydes could be detected in BAL fluid obtained from exercising smokers and nonsmokers exposed twice to ozone (0.22 parts per million [ppm]) and once to filtered air for four hours. Bronchoalveolar lavage fluid was collected immediately after ozone exposure ceased on one occasion and 18 hours after exposure ceased on the other occasion (Frampton et al. 1997). Lavage fluid was collected either immediately or 18 hours after exposure to filtered air and considered to be a single treatment. Pryor and collaborators received coded BAL fluid samples from Frampton that did not identify subjects or exposure protocols. They prepared aldehyde derivatives by reacting the BAL fluid (which had been stored at –80°C) with pentafluorobenzylhydroxylamine hydrochloride. The level of aldehyde derivatives was analyzed by gas chromatography using electron capture detection, as described in the Investigators' Report. After the analytical procedures were completed, the data were sent to Frampton's laboratory, where the codes were identified and the statistical analyses performed.

The derivatization and analytic procedures used in this study are sensitive techniques suited for these analyses. However, a drawback to the study design was that the procedures for determining aldehyde levels were not rigorously examined. For example, the efficiency of the derivatization procedure was not reported, and the gas chromatographic analysis of the aldehyde derivatives was not quantified. Thus, the study's results must be considered as qualitative rather than quantitative.

* Dr. Pryor's Project Report, *Pulmonary Biomarkers for Ozone Exposure*, is available from HEI on request.

† A list of abbreviations appears at the end of the Investigators' Report.

§ Drs. Mark W. Frampton and William A. Pryor's collaborative one-year study began in 1994 with total expenditures of $26,690. The Investigators' Report from Drs. Frampton, Pryor, and colleagues was received for review in July 1998. A revised report, received in December 1998, was accepted for publication in January 1999. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Critique.

RESULTS AND INTERPRETATION

The investigators reported a statistically significant increase in the level of nonanal in BAL fluid obtained from 27 subjects immediately after ozone exposure ceased compared with BAL fluid obtained from the same subjects exposed to filtered air. They ascribe this finding to ozonation of lipids in the epithelial cell lining fluid. Nonanal levels returned toward control levels 18 hours after exposure ceased. (Changes in hexanal were not statistically significant at either time point.) The finding of an increased level of nonanal in human subjects agrees with Pryor and colleagues' observation that nonanal levels increased in BAL fluid from rats exposed to a range of 0.5 to 10 ppm ozone compared with control rats that breathed clean air (Pryor et al. 1996, 1999). In the studies with rats, Pryor and colleagues also reported increases in hexanal and heptanal, which may be due to the ozone exposure levels that were higher than those in the study with humans (these results are presented in the Appendix to the Investigators' Report). An alternative explanation for the difference in the hexanal response between rats and humans is that the number of human BAL fluid samples may have been too small to show a significant increase in hexanal.

The nonanal levels in BAL fluid did not differ between ozone-exposed smokers and nonsmokers. The authors acknowledge this to be a surprising finding; one would expect smokers to have lower nonanal levels because their lungs produce increased amounts of mucus, which may protect them from elevated levels of oxidants. However, the investigators point out that, in the earlier study (Frampton et al. 1997), the degree of airway inflammation induced by ozone exposure also did not differ between the same groups of smokers and nonsmokers. The findings in this pilot study support those of Hamilton and coworkers (1996, 1998) as well, who identified the aldehyde 4-hydroxynonenal in BAL fluid from exercising humans exposed to 0.4 or 0.25 ppm ozone for one hour.

These investigators' findings are important; they suggest that certain aldehydes formed as oxidation products of unsaturated fatty acids may be useful biomarkers of ozone exposure. (Nonanal is also a potentially toxic intermediate that may play a role in adverse effects caused by ozone exposure.) However, because the subjects in this study were exposed to only one concentration of ozone, studies using a range of ozone levels are required to confirm this preliminary observation and substantiate the relation between nonanal formation and ozone exposure level.

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