HEI HEALTH EFFECTS INSTITUTE

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

Part III. Effects on Complex Carbohydrates of Lung Connective Tissue

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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 65 Part III

Changes in Complex Carbohydrate Content and Structure in Rat Lungs Caused by Prolonged Ozone Inhalation

BACKGROUND

Ozone is formed when emissions from mobile and industrial sources react in the presence of sunlight. It is a highly reactive gas, and the major component of urban smog. Because inhaling ozone affects lung function in humans, the U.S. Environmental Protection Agency has set a National Ambient Air Quality Standard (NAAQS) for this pollutant. The current NAAQS is 0.12 parts per million (ppm); compliance requires that this level not be exceeded for more than one hour, once per year.

Because of ozone's reactive nature, prolonged or repeated exposure to ozone is thought to be a potential risk factor for lung cancer. This concern prompted the National Toxicology Program (NTP) to conduct a bioassay of prolonged exposure to evaluate ozone's carcinogenicity in rats and mice. Another concern is that long-term exposure to ozone may injure the tissues of the respiratory tract, and lead to the development or exacerbation of chronic lung diseases such as fibrosis and emphysema. To examine this hypothesis, the NTP allotted additional animals to investigators funded by the Health Effects Institute to study noncancerous alterations in lung tissue structure and function that are characteristic of chronic lung diseases.

One characteristic of the early stages of chemically induced fibrosis and emphysema in laboratory animals is an increase in the level of complex carbohydrates (a heterogeneous group of carbohydrate-containing polymers) in lung connective tissue. Connective tissue is important because it confers mechanical strength to the lungs by providing a supporting framework for cells; however, increased connective tissue can distort normal lung structure and decrease the lung's efficiency for gas exchange.

The study of the effects of long-term ozone exposure on lung complex carbohydrates, described in this report, was one of eight laboratory studies supported by the NTP/HEI collaborative agreement. In addition to studying lung and nasal structure and function, investigators studied other constituents of lung connective tissue.

APPROACH

Dr. Bhandaru Radhakrishnamurthy used standard biochemical methods to measure changes in the content, structure, and function of complex carbohydrates in lung tissue from male and female rats exposed to 0, 0.12, 0.5, and 1.0 ppm ozone for 20 months.

RESULTS AND IMPLICATIONS

Dr. Radhakrishnamurthy found that the total content of complex carbohydrates decreased in lung tissue from rats exposed to the two higher ozone concentrations (0.5 and 1.0 ppm). This loss was accounted for by decreased levels of three of the six complex carbohydrates measured. Structural studies indicated that the size of one complex carbohydrate in ozone-exposed animal tissues was smaller than it was in tissues from control animals. The study also found a trend toward an increase in the level of one complex carbohydrate with increasing ozone concentrations; however, the biological significance of this change is unclear in light of the decreased content of other complex carbohydrates.

The decrease in total complex carbohydrates in this study differs from the results seen in other animal models of fibrosis and emphysema. However, a reactive gas such as ozone may have a different effect on lung tissue than the chemicals used to induce fibrosis or emphysema. Dr. Radhakrishnamurthy's overall results suggest that inhaling ozone at levels of 0.5 ppm or higher may alter the content and size of important connective tissue constituents and may adversely affect the support provided by lung connective tissue.

This Statement, prepared by the Health Effects Institute (HEI) and approved by its Board of Directors, is a summary of a research study sponsored by HEI from 1991 to 1993. The inhalation component of this project was supported by the National Toxicology Program as part of its studies in the toxicologic and carcinogenic effects of ozone. This study was conducted by Dr. Bhandaru Radhakrishnamurthy of the Tulane University School of Public Health and Tropical Medicine, New Orleans, LA. The following Research Report contains an Introduction to the NTP/HEI Collaborative Ozone Project, the detailed Investigator's Report, and a Commentary on the study prepared by the Institute's Health Review Committee.

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INTRODUCTION

The National Toxicology Program and Health Effects Institute Collaborative Ozone Project

The NTP/HEI Collaborative Ozone Project was a four-year project with many investigators that was organized to evaluate the effects of prolonged ozone exposure on lung injury in animals. The ozone exposures were conducted by the National Toxicology Program (NTP) at Battelle Pacific Northwest Laboratories. The individual investigators' studies, which addressed the pathologic and physiologic consequences of prolonged ozone exposure, were supported by the Health Effects Institute (HEI). A full description of the NTP/HEI Collaborative Ozone Project and the exposure protocol can be found in the Introduction and Supplement to Research Report Number 65 Part I. This information also will be published in Part VI of Research Report Number 65 that describes the exposure and distribution of the animals.

Briefly, in 1987, the Health Effects Institute entered into a partnership with the National Toxicology Program to evaluate the effects of chronic ozone exposure in rats. The NTP, consisting of four agencies of the U.S. Department of Health and Human Services, coordinates the nation's testing of potentially toxic and hazardous chemicals. The Health Effects Institute, an independent research organization supported by both government and industry, provides unbiased information on the health effects of motor vehicle emissions.

Because of the widespread exposure to ozone and concerns about its potential health effects, HEI and the California Department of Health and Human Services nominated ozone for carcinogenicity and toxicity testing by the NTP. The NTP, recognizing that cancer was only one of the chronic diseases of concern, included additional animals for HEI-supported studies of the pathologic and physiologic consequences of prolonged ozone exposures. The HEI animals were housed in cages that would otherwise have been empty. By developing a partnership, the HEI and NTP were able to leverage their funds to develop a comprehensive research program that extended beyond carcinogenicity endpoints; the HEI-sponsored research focused on the relation between long-term ozone exposure and the pathogenesis of chronic lung diseases, such as asthma, emphysema, and fibrosis. The Health Effects Institute would not have been able to undertake such an expensive project, which requires special facilities and trained personnel, without the NTP's support of the inhalation component and the cooperation of the NTP's contractor, Battelle Pacific Northwest Laboratories.

For the HEI component of the Project, eight studies were selected for funding from proposals submitted in response to the Request for Applications (RFA) 90-1, "Health Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program–Health Effects Institute Studies, Part A: Respiratory Function Studies, and Part B: Structural, Biochemical, and Other Alterations." Because of the complexity of a project with many investigators and many endpoints, the HEI Health Research Committee also funded a Biostatistical Advisory Group to provide assistance with experimental design, animal allocation, and data analyses. Figure 1 presents a diagram of the studies in the NTP/HEI Collaborative Project and their relations to each other. They include those studies that were part of the NTP bioassay, the eight HEI-funded studies and the biostatistical study. In addition, HEI engaged Battelle Pacific Northwest Laboratories to provide support services for the HEI-sponsored investigators.

Starting at six to seven weeks of age, male and female F344/N rats were exposed to 0, 0.12, 0.5, or 1.0 parts per million (ppm) ozone, 6 hours per day, 5 days per week. These concentrations were selected to include the maximum concentration the animals would tolerate (1.0 ppm), the current National Ambient Air Quality Standard (NAAQS) for ozone (0.12 ppm), and an intermediate concentration. The NTP's carcinogenicity bioassay consisted of a two-year study and a lifetime study in rats and mice, and a study of male rats exposed to 0.5 ppm ozone and two levels of a human pulmonary carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The design of the HEI studies was directed, to some extent, by the constraints of the NTP protocol. These included ozone exposure concentrations that were set by the NTP, a limit on the sample size (164 rats) to the number of available exposure chambers, and quarantine restrictions that did not allow reentry of animals into the exposure chambers once they had been removed, thus eliminating the possibility of conducting serial tests.

The Biostatistical Advisory Group developed a sample allocation scheme that allowed several researchers to obtain measurements on tissue samples from the same subset of study animals, providing the maximum overlap of animals and tissues among the eight studies while ensuring balance with respect to dose, gender, and time of death. When the ozone exposures for the HEI animals ended (at 20 months), several investigators traveled to Battelle Pacific Northwest Laboratories to conduct their assays or to obtain samples on site. Battelle personnel prepared the tissues for off-site investigators and shipped them directly to their laboratories.

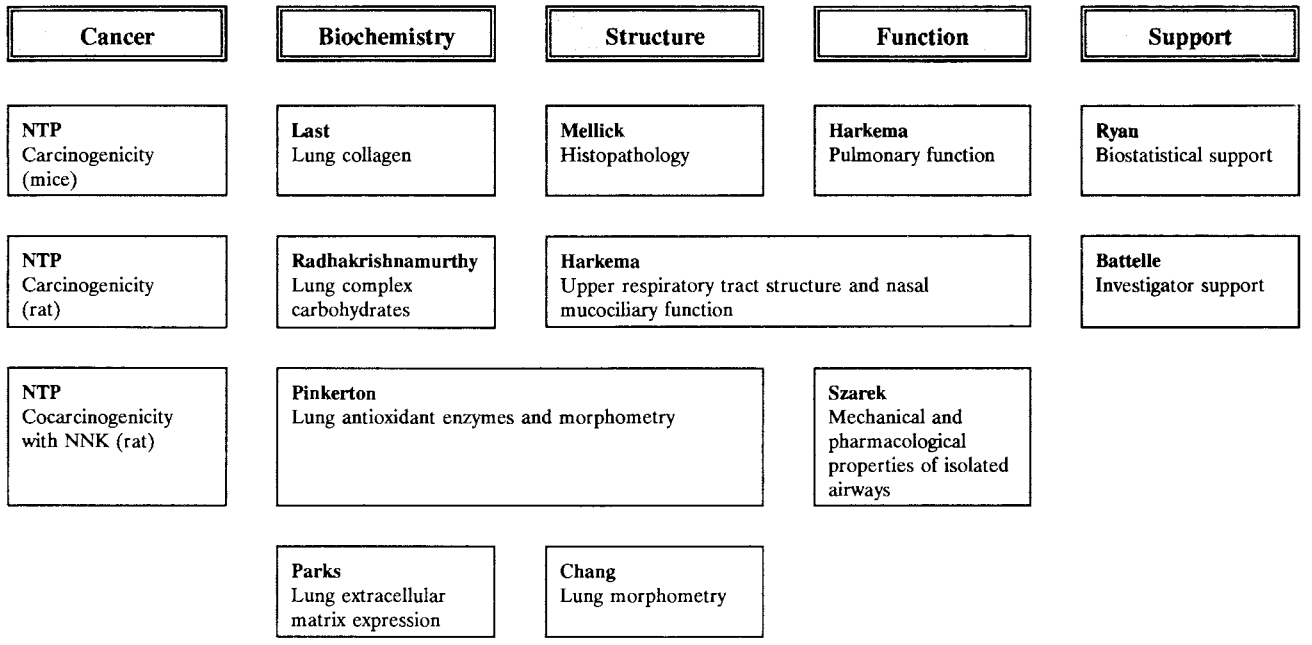

Figure 1. The NTP/HEI Collaborative Ozone Project: Individual Studies.

Because the studies varied in duration from six months to two years, HEI is publishing the reports for each individual study after the Institute's review process for each study is complete. Each Investigator's Report and a forthcoming Integrative Summary Report will be Parts of Report Number 65 of the HEI Research Report series. The present study by Dr. Bhandaru Radhakrishnamurthy of the effects of long-term ozone exposure on the complex carbohydrates of lung connective tissue is Part III. Other investigators in the Collaborative Ozone Project examined the effects of ozone on functional (Harkema, Szarek), structural (Pinkerton, Chang), or biochemical (Last, Parks) alterations.

Although some conclusions can be drawn on the basis of the results from each individual study, the interpretation of Dr. Radhakrishnamurthy's findings will be strengthened when his data are correlated with the outcomes of the other investigators.

The importance of the collaborative NTP and HEI chronic ozone exposure studies is that they provide an unparalleled opportunity to examine the effects of prolonged ozone exposure using a variety of scientific approaches. The interaction of a number of methods to analyze the pathologic and physiologic consequences of chronic ozone exposure is one of this project's unique features. The results of these studies will provide new information about the threshold effects of ozone exposure on lung injury and the type and extent of damage in a well-established animal model. These results may be helpful for evaluating current standards of ozone exposure as they apply to human health and for designing future animal and human studies.

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

Part III: Effects on Complex Carbohydrates of Lung Connective Tissue

Bhandaru Radhakrishnamurthy

ABSTRACT

Glycosaminoglycans are constituents of proteoglycans, which are integral components of lung connective tissue. Glycosaminoglycans not only provide structural support to organs, but also influence extracellular matrix assembly, cell adhesion, and cell proliferation. Changes in the metabolism of glycosaminoglycans have been noted in several pulmonary diseases, for example, pulmonary fibrosis and emphysema.

We studied quantitative and qualitative changes of glycosaminoglycans in the lungs of rats exposed to a range of ozone levels (0, 0.12, 0.5, 1.0 parts per million) for 20 months. Glycosaminoglycans were isolated from dry-defatted lung tissues through successive digestions by pronase, papain, and 2 M sodium hydroxide. The glycosaminoglycans then were fractionated into individual components using high-performance liquid chromatography. The concentration of total glycosaminoglycans in the tissues varied from 1.5 to 4.2 μg of uronate/mg of dry-defatted tissue. Although wide variations in total glycosaminoglycan concentrations exist among individual animals within each exposure group, regression analyses of data indicate a monotonic and statistically significant decrease of total glycosaminoglycans after ozone exposure ($p = 0.02$).

Among individual glycosaminoglycans, hyaluronan, chondroitin 4-sulfate, and chondroitin 6-sulfate levels decreased significantly ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively) in animals exposed to ozone when compared with control animals. Heparan sulfate concentration exhibited a significant ($p < 0.05$) trend toward increase with increasing doses of ozone, but the difference in heparan sulfate concentration between ozone-exposed animals and control animals was not significant.

Gel filtration studies of glycosaminoglycans in pooled samples indicated that the molecular size of hyaluronan in animals exposed to ozone was lower than it was in control animals. We noted differences in heparan sulfate's chemical properties and affinity to antithrombin III in ozone-exposed animals and control animals. Although these studies do not provide the mechanism responsible for the observed changes in the lung glycosaminoglycans in ozone-exposed animals, the observations indicate that inhalation of ozone for 20 months affects normal cellular metabolism of proteoglycans, which may contribute to the functional impairment of the lung.

INTRODUCTION

Ozone, a major constituent of photochemical smog, is one of six criteria air pollutants (U.S. Environmental Protection Agency 1986). Ozone is formed through complex photochemical reactions of nitrogen oxides and volatile organic emissions from automobiles. Considerable data from clinical, epidemiologic, and animal studies show that chronic inhalation of ozone causes injury to the respiratory tract (Lippmann 1989; Tilton 1989). Animal studies indicate that both acute and chronic exposure to ozone results in biochemical changes, cellular injury, and structural alterations in the lung (Freeman et al. 1973; Penha and Werthamer 1974; Freeman and Crapo 1982). Although the connective tissue framework is important in the lung, little is known about ozone's effect on this system's components, especially proteoglycans.

Proteoglycans are polyanionic macromolecules that consist of a protein backbone to which sulfated glycosaminoglycan (GAG) chains are covalently attached. Hyaluronan

* 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 65, Part III, which also includes an Introduction to the NTP/HEI Collaborative Ozone Project, 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. Bhandaru Radhakrishnamurthy, Department of Applied Health Sciences, Tulane University School of Public Health and Tropical Medicine, 1430 Tulane Avenue SL29, New Orleans, LA 70112.

This study was supported by HEI funds from the U.S. Environmental Protection Agency and the motor vehicle industry. The inhalation component of this project was sponsored by the National Toxicology Program as part of its studies in the toxicologic and carcinogenic effects of ozone.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 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 view of the Agency, and no official endorsement should be inferred. The contents of this documnent 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.

(HA) is the only GAG that occurs in the native state without covalent attachment to a protein. Proteoglycans' biologic properties include structural and supportive functions to the body and organ systems, hemostasis and thrombosis, control of fluid and electrolytes, lubrication and shearing activities, viscoelasticity, inflammation and repair, regulation of membrane permeability, cell to cell and cell to extracellular matrix interactions, resistance to infection, and an influence on fibrillogenesis (Berenson et al. 1984). Some of these properties are important factors in the development of lung disease. The metabolism of proteoglycans is regulated by hormonal, nutritional, genetic, and environmental factors (Kennedy 1979; Varma and Varma 1982). Changes in the composition of the lung GAGs are noted in several pathological conditions (Motomiya et al. 1982; Radhakrishnamurthy and Berenson 1989; Juul et al. 1991).

SPECIFIC AIMS

Because ozone is a powerful oxidant and affects the metabolism of proteins, lipids, and carbohydrates, it is hypothesized that ozone inhalation alters the composition of GAGs. Our strategy was to (1) isolate and quantify total GAGs from the lungs of rats exposed to ozone at different concentrations, (2) fractionate GAGs into individual types, and (3) observe changes in their composition resulting from ozone exposure.

METHODS AND STUDY DESIGN

ANIMALS

Exposure to ozone was performed at Battelle Pacific Northwest Laboratories (Richland, WA) as part of a collaborative, multilevel study with the National Toxicology Program (NTP) and the Health Effects Institute (HEI) to examine the long-term effects of inhaled ozone. Four- to five-week-old male F344/N rats were obtained from Simonsen Laboratories (Gilroy, CA). Rats were quarantined for 10 to 14 days then randomly assigned to ozone exposure or to filtered air (control groups). Rats were housed individually in stainless-steel wire-bottom cages in modified Hazelton 2000 inhalation chambers. Exposures were for six hours per day (between 7:30 a.m. and 5:30 p.m.), five days per week, for 20 months. NIH-07 open formula pelleted diet (Zeigler Bros., Inc.; Gardners, PA) was available ad libitum except during exposure periods. Softened tap water (city of Richland water supply) was delivered ad libitum except during exposure by an automatic watering system. Animal rooms were provided with timer-controlled fluorescent lighting on a 12-hour on/12-hour off cycle with the lighted period beginning at 6:00 a.m. Rats were exposed to one of three concentrations of ozone: 0.12, 0.5, or 1.0 parts per million (ppm). The temperature of the chambers was maintained at 75°F and relative humidity at 57% to 58%. The rate of air flow was 15 ft^3/min. In these experiments we followed the Standards established by the Animal Welfare Act of 1985 and the 1985 revised edition of the Public Health Service document entitled "Guide for the Care and Use of Laboratory Animals."

TESTS FOR RESPIRATORY INFECTIONS

Sera of rats not used for experimental determinations (sentinal rats) were tested for rodent pathogens at 3 weeks, 6 months, 12 months, 18 months, and when they were killed at the end of the study. Pathogens tested for included H-1 virus, Kilham rat virus, *Mycoplasma arthritides*, *Mycoplasma pulmonis*, pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, and Sendai virus.

OZONE GENERATION AND MONITORING

Ozone was generated from 100% pure oxygen using a silent-arc (corona) discharge ozonator (OREC Model 03V5-0, Phoenix, AZ). Ozone concentration in each chamber was monitored by a multiplexed Dasibi Model 1003-AH (Dasibi Environmental Corporation, Glendale, CA) ultraviolet spectrophotometric analyzer. The monitor was calibrated by comparison with a chemical-specific monitor (calibrated using the neutral-buffered potassium iodide method) that simultaneously sampled exposure chambers. Chamber uniformity was measured periodically at 12 levels or locations in each chamber. A recirculation device enhanced the uniformity of ozone concentration within each chamber. Potassium permanganate filters extracted ambient ozone from air entering all chambers. Charcoal and high-efficiency particulate air (HEPA) filters provided additional filtration of air entering the exposure chambers. The chamber airflow rate was set to provide 15 air changes per hour.

TISSUES

Following necropsy at Battelle Pacific Northwest Laboratories, lung tissues were dissected from extraneous tissues, then quickly rinsed three times with cold (4°C) 0.15 M sodium chloride (NaCl) to remove blood from the tissue. Excess water was removed by blotting with a filter paper. Then the tissue was weighed, frozen, packed over dry ice, and shipped from Richland, WA to New Orleans, LA by overnight air freight.

In this study, 47 lung tissues were used, 27 from right caudal lung lobes and 20 from accessory lobes. We used 14 tissues from control animals, 6 from rats exposed to 0.12 ppm ozone, 13 from rats exposed to 0.5 ppm ozone, and 14 from rats exposed to 1.0 ppm ozone. Males and females were represented equally in each group except in the group exposed to 0.5 ppm ozone, in which there were seven females and six males. The animals were killed on three consecutive days after the cessation of exposure to ozone: 1 on day 6 because of its poor condition following pulmonary function tests, 26 on day 7, and 20 rats on day 8.

ANALYTICAL PROCEDURES

Uronic acid determinations were done by the carbazole–sulfuric acid (H_2SO_4) reaction described by Dische (1947) as modified by Bitter and Muir (1962). In samples in which there was interference in the Bitter-Muir reaction, we attempted to determine uronic acid by 3-phenylphenol–H_2SO_4 reaction (Blumenkrantz and Asboe-Hansen 1973). Hexosamine was assayed by the method of Boas (1953) after the sample had been hydrolyzed in 4 N hydrochloric acid (HCl) for 14 to 16 hours at 100°C. Total sulfate was determined by the procedure of Terho and Hartiala (1971) using sodium rhodizonate. *N*-Sulfate analysis was done by the method of Lagunoff and Warren (1962).

Glucuronic acid and iduronic acid proportions in heparan sulfate (HS) fractions were determined by high-performance liquid chromatography (HPLC) using the Dionex System (Sunnyvale, CA). Prior to chromatography HS fractions were hydrolyzed by formic acid at 100°C for 14 to 16 hours (Radhakrishnamurthy et al. 1968). The chromatography was performed on Carbopac PA1 column with 100 mM sodium hydroxide (NaOH) and 150 mM sodium acetate as eluant at a flow rate of 1 mL/min and with pulsed amperometric detection (PAD, Gold, Dionex Technical Note 20).

Isomeric chondroitin sulfates were determined by the method of Saito and colleagues (1968) using chondroitinases ABC and AC, and chondroitin 4- and 6-sulfatases. The GAGs were incubated with chondroitinase ABC or AC and the appropriate sulfatase for 1 hour at 37°C. Unsaturated disaccharides released by the enzymes in the incubation mixtures were quantified by *N*-acetylgalactosamine determination. Chondroitin 4- and 6-sulfate (C4-S, C6-S) and dermatan sulfate (DS) in the GAG mixture were estimated from the relative proportion of unsaturated disaccharides.

ISOLATION OF GLYCOSAMINOGLYCANS

The lung tissues were dried and defatted simultaneously by treatment with acetone (5 mL/mg wet tissue) (hereafter referred to as dry-defatted tissue). Acetone was decanted after 24 hours and fresh acetone was added; this procedure was repeated three times. The tissues were dried to a constant weight in a vacuum desiccator.

Glycosaminoglycans were isolated from a known amount of dry-defatted tissue according to previously described procedures (Rodén et al. 1972; Seethanathan et al. 1975). The tissues were first digested three times with papain; the residual tissue was digested three times with pronase. The digestion by papain was carried out at 65°C for 48 hours in 0.1 M phosphate buffer, pH 6.5, containing 0.005 M ethylene diaminotetraacetic acid (EDTA) disodium salt, and 0.005 M cystein hydrochloride. The pronase digestion was done at 37°C for 48 hours in 0.1 M phosphate buffer pH 7.8. After pronase, undigested tissue was digested with 2.0 M NaOH at 24°C for 48 hours. To remove protein materials from GAGs, the digests were pooled, dialyzed against distilled water, deproteinized through a Dowex $50H^+$ column (0.9 cm × 15 cm) and lyophilized. The total GAG content in each sample was estimated on the basis of uronic acid content (Bitter and Muir 1962).

FRACTIONATION OF GLYCOSAMINOGLYCANS

The GAG isolates (100 to 200 µg uronate) were fractionated into individual types by an HPLC (Dionex system) procedure (Lee et al. 1987). The chromatography was performed on a TSK GEL DEAE-35W column (TSK-GEL®, Tosohass Corp., Philadelphia, PA) using a NaCl gradient (0 to 3.5 M in 10 % methanol) with the program shown in Table 1.

The column eluate was collected into 2-mL fractions using a fraction collector, and 50 µL of each fraction was monitored for uronic acid (Bitter and Muir 1962). The fractions were pooled into four fractions on the basis of uronic acid profiles. The pooled fractions were dialyzed against distilled water to remove NaCl, then lyophilized and reconstituted in 2.0 mL of distilled water. The concentration of GAGs in each pooled sample was determined by uronic acid (Bitter and Muir 1962) or by hexosamine content (Boas 1953).

Because the amounts of total uronate obtained from 14 tissue samples (four from the control group, four from the 0.5-ppm ozone group, and six from the 1.0-ppm ozone group) were small, they were pooled into groups of two samples in each group for HPLC. Pooling was done on the basis of gender and the day the rat was killed. Altogether,

Table 1. Program for High-Performance Liquid Chromatography of Glycosaminoglycans

Time (minutes)	Composition of Eluant: Pump 1 (10% methanol)	Composition of Eluant: Pump 2 (3.5 M NaCl in 10% methanol)
0-20	100	0
25	85	15
35	75	25
42	68	32
52	66	34
60	55	45
65	50	50

32 GAG preparations were fractionated by HPLC. We did not monitor HPLC fractions by uronate in six samples because the total amounts of uronate were too small. High-performance liquid chromatography fractions from these six samples were pooled into four groups representing HA, HS, CS + DS, and Hep based on standard GAG profiles. Pooled fractions under each peak from each of the 32 samples were dialyzed, lyophilized, and reconstituted in distilled water, then analyzed for uronic acids (Bitter and Muir 1962). However, because eight samples had interference (green color) in the reaction, we analyzed all the samples for hexosamine (Boas 1953) to quantify individual GAGs. For uniformity, the concentrations of individual GAGs in the tissues were expressed based on hexosamine content. Concentrations of isomeric chondroitin sulfates (CS) in the tissues were calculated from the total hexosamine of the C4-S + C6-S + DS fraction and from the relative proportions of C4-S, C6-S, and DS estimated by enzymatic assay (Saito et al. 1968).

As an additional test of identification by HPLC, GAGs were identified in each pooled fraction by cellulose acetate electrophoresis. Results showed no overlapping of GAGs in the fractions within the limits of detection (± 5 percent).

CELLULOSE ACETATE ELECTROPHORESIS

Cellulose acetate electrophoresis was performed in 0.1 M pyridine-formic acid buffer pH 3.0, followed by localization of GAGs by alcian blue (Matalon and Dorfman 1966; Ehrlich et al. 1975).

GEL FILTRATION

Gel filtration of GAGs was performed on a Sepharose CL-2B column (0.9 × 15 cm), which was eluted with 0.05 M sodium acetate buffer pH 5.8, containing 0.15 M NaCl. Fractions of 0.25 mL were collected every two minutes using a fraction collector. Void volume (V_o) and total volume (V_t) of the column were determined using Blue Dextran and glucosamine respectively. Fractions were monitored for GAGs by hexosamine analysis (Boas 1953). K_{av} values of GAGs were calculated by the formula

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where K_{av} is the fraction of gel volume that is available for diffusion of solute, and is proportional to log molecular weight; and V_e is the elution volume of the peak.

ANTITHROMBIN III: AFFINITY CHROMATOGRAPHY

Agarose-antithrombin III gel matrix (Sigma, St. Louis, MO) was used to study antithrombin III binding of HS fractions (Höök et al. 1976). Heparan sulfate fractions were dialyzed against 0.1 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) HCl buffer (pH 7.4) and applied to the column of immobilized antithrombin III (3 mL) previously equilibriated with the buffer at 4°C. The column was washed with the buffer (24 mL) until the effluent was free from HS as determined by hexosamine analysis (Boas 1953). The column was then eluted with the Tris-HCl buffer containing 2.5 M NaCl (25 mL) to obtain HS that was bound to the column. The eluates were dialyzed to remove salts, then lyophilized, reconstituted in 1.0 mL distilled water, and analyzed for hexosamine (Boas 1953). Low-affinity HS fractions did not bind to antithrombin and eluted from the column in the wash buffer. High-affinity HS fractions reacted with antithrombin III and eluted from the column by treatment with 2.5 M NaCl.

STATISTICAL METHODS AND DATA ANALYSIS

Glycosaminoglycan determinations were done on coded samples that could not be identified by the technicians. After completing the measurement, the samples were decodified and the data subjected to analysis of variance (ANOVA) and linear regression analysis (Snedecor and Cochran 1989). Dunnett's criterion (Glantz et al. 1990) was used to compare each exposure level with control animals. In addition to pairwise comparisons, tests for trend with ozone were conducted for all endpoints (Snedecor and Cochran 1989). Statistical significance level was $p < 0.05$.

RESULTS

The mean body weight of the rats exposed to 1.0 ppm ozone (398.4 g) was slightly, but not significantly, lower than the mean body weight of control rats (432.5 g). There were no differences in body weights between rats exposed to 0.12 or 0.50 ppm ozone and control animals. The sera from sentinal rats were negative for respiratory pathogens. The mean wet weight of the right caudal lobes used in the study was 0.39 g; the mean wet weight of the accessory lobes was 0.24 g. Acetone dry-defatted weights of the tissues represented 15% of their wet weights. Glycosaminoglycans were isolated from each of the 47 dry-defatted tissues and quantified by uronic acid determination. We could not determine uronic acid in five samples because in the carbazole–H_2SO_4 reaction (Bitter and Muir 1962) and in the 3-phenylphenol–H_2SO_4 reaction (Blumenkrantz and Asboe-Hansen 1973) intense green color developed rather than pink. The development of green color in these reactions could be due to contaminants such as metals (Fe^{+2} for example) or substances that could be oxidized by H_2SO_4. It is unlikely that the GAG isolates were contaminated by protein materials because protein materials turn brown in the uronic acid reactions used in the study. We did not trace the exact nature of the contaminants in these GAG preparations.

The concentration of total GAGs, expressed as micrograms of uronic acid per milligram of dry-defatted tissue, from each of the ozone groups and control animals are shown in Table 2. Although total concentration of GAGs varied widely even within the groups, 1.0 ppm ozone exposure reduced the mean (± SE) concentration of GAGs from 3.09 ± 0.16 to 2.42 ± 0.18 in the tissues ($n = 13$ in both groups), and the difference was statistically significant ($p = 0.01$, Figure 1). There was no significant difference in total concentration of GAGs between male (mean 2.7) and female (mean 2.6) rat tissues. Similarly, no significant difference was apparent in concentration of GAGs in tissues of rats killed on day seven (2.7) and day eight (2.6) following cessation of ozone exposure. However, a significant difference ($p = 0.02$) in concentration of GAGs was seen between right caudal lobes and accessory lobes. The mean (± SE) concentration of GAGs in the right caudal lobes was 2.46 ± 0.13, and the accessory lobes had a mean concentration of 2.94 ± 0.12. Total levels of GAGs show a monotonic decrease with ozone concentration (Figure 2). This trend was confirmed by regression analysis in which a significant negative relationship ($p = 0.02$) was found (Table 3).

Figure 3 shows HPLC profiles of GAG standards and GAGs from four lung tissues as examples. The procedure provides fractions containing HA, HS, C4-S + C6-S + DS, and heparin (Hep) from mixtures of GAGs (Lee et al. 1987). These glycosaminoglycan profiles were similar to those of standard GAGs. The reproducibility of quantitation of individual GAGs by this procedure was checked 20 times using the same mixture of GAGs on 10 consecutive days. Both within-day and day-to-day variations were noted. For within-day variations, the coefficient of variation for individual GAGs ranged from 0 to 6%. For day-to-day variation, values ranged from 2.7 to 7.8%. The results indicated good reproducibility in the procedure.

Figure 4 illustrates the concentrations of individual GAGs in the lung samples. The concentrations of individual GAGs within each ozone exposure group showed variations similar to those seen in the concentration of total GAGs. Hyaluronan and HS were the major GAGs in the tissue, constituting about 63% of the total GAGs in the control tissue. The other GAGs constituted 4% to 10% each. In the tissue from animals exposed to 0.5 and 1.0 ppm ozone, HA decreased significantly ($p < 0.05$ and 0.001 respectively). Although heparan sulfate increased with exposure to 0.5 and 1.0 ppm ozone, and the trend test (Snedecor and Cochran 1989) was statistically significant ($p < 0.05$), no single exposure group was increased significantly relative to the control group. Among minor GAGs, C4-S and C6-S showed significant decreases at 0.5 and 1.0 ppm ozone exposure. Measurements of DS and Hep from animals exposed to 0.5 or 1.0 ppm ozone did not differ significantly from control animals. With the exception of Hep, the differences between the animals exposed to 0.12 ppm ozone and control animals were not statistically significant. However, the number of animals in the group exposed to 0.12 ppm was too small to draw conclusions. Although data are not shown in the figure, the concentrations of individual GAGs did not differ significantly between genders and between the days rats were killed. Remaining samples were pooled and used for gel filtration studies.

Figure 5 illustrates the gel filtration profiles for HA, HS, and C4-S + C6-S + DS from pooled fractions from control animals and from animals exposed to 0.5 ppm or 1.0 ppm ozone. The molecular size of HA from animals exposed to 1.0 ppm ozone was smaller than the molecular size of HA from control animals ($K_{av} = 0.24$ versus 0.18). The peak of HA from animals exposed to 0.5 ppm ozone was broader than that from control animals. There was no difference in K_{av} values of HS ($K_{av} = 0.47$) and CS + DS ($K_{av} = 0.41$) peaks between control animals and ozone-exposed animals.

Table 4 shows analyses of HS fractions from control and ozone-exposed animals. These analyses also were performed on pooled samples. The proportion of iduronic acid to glucuronic acid was less in ozone-exposed animals

Table 2. Total Concentration of Glycosaminoglycans in Lung Tissues from Control and Ozone-Exposed Rats

Rat Number (H Series)	Gender	Days Following the Cessation of Ozone Exposure[a]	Lung Lobe[b]	Total GAG (μg uronic acid/mg dry-defatted tissue)
Control Animals (Exposed to 0.0 ppm Ozone)				
1	M	8	RCL	ND[c]
4	F	8	RCL	3.5
7	M	7	AL	3.7
10	F	7	AL	3.2
25	F	7	RCL	3.4
28	M	7	RCL	3.1
31	F	7	RCL	3.2
34	M	8	RCL	2.8
133	M	7	RCL	2.0
137	F	8	RCL	2.9
149	M	7	AL	4.2
153	F	8	AL	2.3
157	F	8	AL	2.7
161	M	7	AL	2.7
Animals Exposed to 0.12 ppm Ozone				
134	M	7	RCL	2.2
138	F	8	RCL	2.3
150	M	7	AL	2.3
154	F	8	AL	3.3
158	F	8	AL	2.7
162	M	7	AL	2.8
Animals Exposed to 0.5 ppm Ozone				
2	M	6	RCL	1.7
5	F	8	RCL	2.4
8	M	7	AL	2.8
11	F	7	RCL	3.4
26	F	7	RCL	2.7
29	M	7	RCL	NDc
32	F	7	RCL	1.7
35	M	8	RCL	2.6
135	M	7	RCL	ND[c]
139	F	8	RCL	2.0
155	F	8	AL	3.3
159	F	8	AL	ND[c]
163	M	7	AL	2.6
Animals Exposed to 1.0 ppm Ozone				
3	M	8	RCL	1.8
6	F	8	RCL	1.5
9	M	7	AL	2.7
12	F	7	AL	3.6
27	F	7	RCL	2.4
30	M	7	RCL	1.9
33	F	7	RCL	1.5
36	M	8	RCL	2.0
136	M	7	RCL	2.9
140	F	8	RCL	3.2
152	M	7	AL	2.5
156	F	8	AL	3.0
160	F	8	AL	ND[c]
164	M	7	AL	2.6

[a] Number of days animals were exposed to filtered air after the cessation of ozone exposure and before necropsy; some control animals were killed along with ozone-exposed animals.

[b] Two types of lung tissues were used in these studies: right caudal lobe (RCL) and accessory lobe (AL).

[c] ND = not determined because of interference in the carbozole-H_2SO_4 reaction.

than in control animals. Total sulfate and N-sulfate contents in HS from ozone-exposed animals were lower than in HS from control animals. The relative proportions of high-affinity to low-affinity binding to antithrombin III of HS fractions from ozone-exposed animals were lower than those from control animals.

DISCUSSION AND CONCLUSIONS

We observed that chronic exposure to ozone decreases the concentration of total GAGs in lung tissues. It is unclear whether the decrease is due to decreased synthesis or increased degradation. The biosynthesis of GAGs or proteoglycans involves the concerted activities of several glycosyltransferases (Rodén 1980). If any of these enzyme activities are affected by ozone inhalation, a change in GAG synthesis occurs. Also, a protein core with appropriately placed serine residues is needed for proteoglycan synthesis. If ozone affects the core protein structure, the synthesis of proteoglycans can be altered. Ozone inactivates protease inhibitors (Riley and Kerr 1985) and uninhibited activities of proteases and glycosidases also can contribute to a decrease in total GAG concentration in tissues from ozone-exposed animals.

We observed that HA constituted about 37% of the total GAGs in control rat lung tissue. In other studies, HA constituted lower percentages of total GAGs: 3% in hamster lung tissue (Karlinsky 1982); 12% in rabbit lung tissue (Horowitz and Crystal 1975); and 23% in human lung tissue (Konno et al. 1982). It is unclear whether variations in HA concentration are due to differences in species or methodology. Karlinsky (1982) and Horowitz and Crystal (1975) used defined parenchymal tissues in their studies, whereas we used specific lobes. In their study, Konno and associates (1982) used whole lung tissues. Furthermore, Karlinsky (1982) quantified GAGs by an electrophoretic procedure; Horowitz and Crystal (1975) used enzymatic

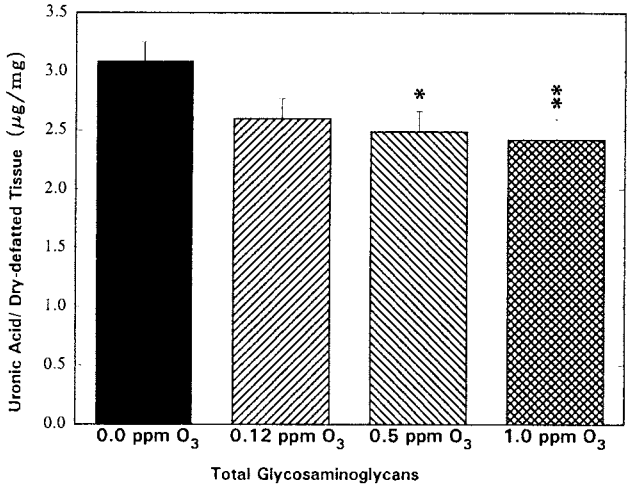

Figure 1. Concentration of total GAGs in lung tissues from rats exposed to different concentrations of ozone. The five samples in which we could not determine uronic acid are not included. The p values (* = $p < 0.05$; ** = $p < 0.01$) correspond to pairwise t tests comparing each dose group to the control group. Control, $n = 13$; exposure to 0.12 ppm ozone, $n = 6$; exposure to 0.5 ppm, $n = 10$; exposure to 1.0 ppm, $n = 13$.

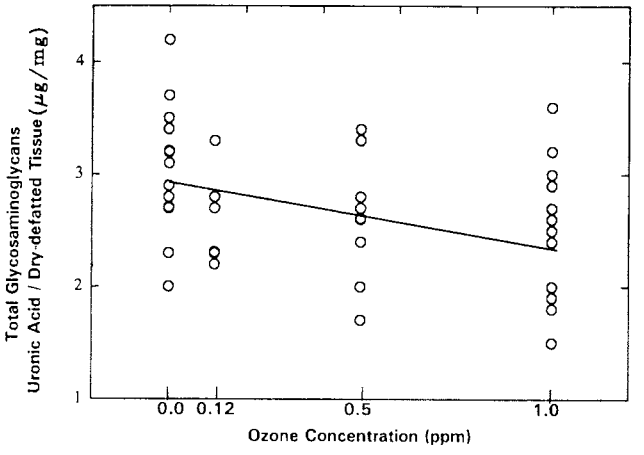

Figure 2. Observed and fitted regression data for concentration of total GAGs in lung tissues after exposure to 0, 0.12, 0.5, or 1.0 ppm ozone.

Table 3. Regression of Glycosaminoglycans[a] by Ozone, Gender, Day Rat Was Killed, and Lobe Type

Glycosaminoglycans	Coefficient	SE	t[b]	p Value[c]
Ozone exposure level	−0.51	0.20	−2.51	0.02
Gender	0.13	0.09	1.41	0.17
Day rat was killed	−0.12	0.18	−1.22	0.23
Lobe type	−0.21	0.09	−2.45	0.02

[a] Glycosaminoglycans were normalized by micrograms of uronic acid per milligram of dry-defatted tissue. See Table 2 for raw data.

[b] Student t test.

[c] Level of significance.

procedures; and we used an HPLC procedure. The methods of isolating GAGs differed as well. Horowitz and Crystal (1975) used cetylpyridinium chloride to precipitate GAGs from the tissue extracts; we directly isolated GAGs from the extracts after removing proteinous material by exhaustive dialysis of the extract and then passing it through a Dowex-$50H^+$ column.

Glycosaminoglycan changes are an important early response to different forms of lung injury. In experimental studies of lung injury based on the nature of the agent used to induce injury as well as the timing of analysis of tissues, both increases and decreases in concentration of GAGs were noted. Involvement of GAGs in emphysema appears to be most demonstrable when early changes are studied. Karlinsky (1982) noted in elastase-induced emphysema that DS synthesis peaked on day 3, followed by peaks in HA and chondroitin sulfate synthesis on day 10. All GAGs returned to baseline by day 21. In pulmonary fibrosis, GAG changes appear to occur prior to collagen accumulation in the diseased lung. In experimental fibrosis, Cantor and associates (1980, 1983) showed that GAG synthesis increased at day 15 and at day 30 after injury, primarily because of an increase in DS. After that, the differences in GAG synthesis between control and experimental tissues were no longer significant.

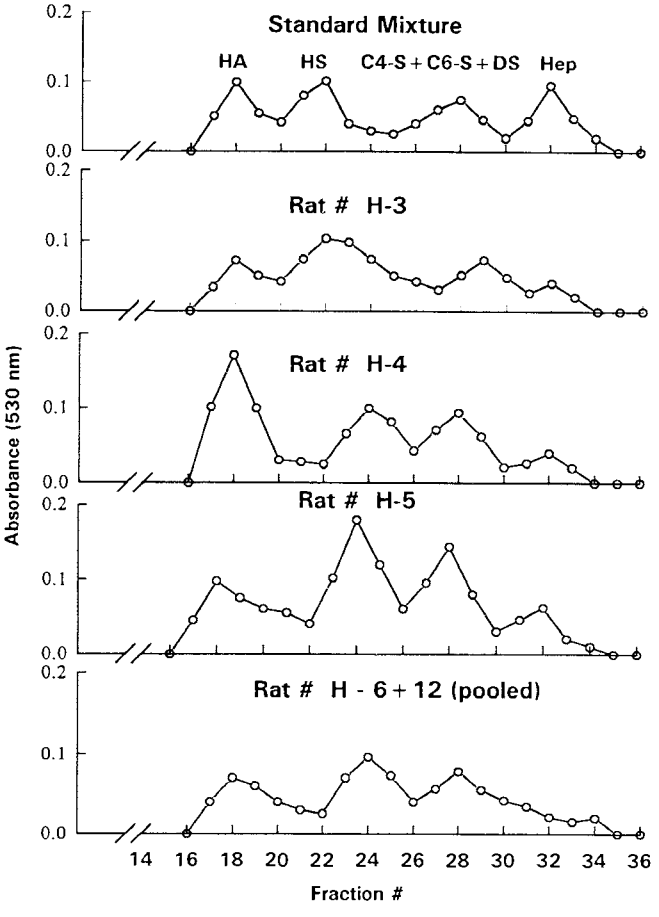

Figure 3. HPLC of a mixture of standard GAGs and GAGs from lung tissues of rats exposed to ozone. A known amount of GAG (100 to 200 μg uronate) was fractionated by HPLC as described in the Methods section. The fractions were monitored for uronate by carbazole-H_2SO_4 reaction. The recoveries of total uronate were 92% to 98% of the material chromatographed. Ozone exposure: rat H-3, 1.0 ppm; rat H-4, 0.0 ppm; rat H-5, 0.5 ppm; rats H-6 and H-12 pooled, 1.0 ppm.

In studies of experimental emphysema, we noted that lungs from pronase-treated rabbits contained considerably greater GAG concentrations than did lungs from control animals (Radhakrishnamurthy et al. 1985). But with cadmium chloride ($CdCl_2$) treatment to induce emphysema, we noted lower GAG concentrations than in control animals, similar to what we observed with ozone in the present investigation. It is likely that ozone, similar to $CdCl_2$ produces biochemical toxicity, interferes with oxidative phosphorylation, and reacts with sulfhydryls of proteins causing alterations in cellular metabolism in the lung.

Our observations in this investigation indicate that ozone exposure alters the concentration of individual GAGs. Significant decreases were noted in the concentrations of HA and CS in ozone-exposed lung tissues. Elevated levels of HA were noted in several lung diseases including severe asthma, mesothelioma, squamous cell lung tumors, adult respiratory distress syndrome, and emphysema (Montomiya et al. 1982). The biochemical mechanisms responsible for the elevation of HA in these diseases are not known. The observed decrease of HA, C4-S, and C6-S in ozone-exposed animals could be due to toxic effects of ozone on one or more cell types that synthesize these GAGs in the lung. The observed lower molecular size and broader peak in gel chromatography of HA in ozone-exposed animals are likely due to oxidative degradation of HA by ozone (Pigman and Rizvi 1959).

Analyses of HS fractions indicate that ozone exposure affects the chemical characteristics of this GAG. Microheterogeneity is a common feature of HS. Heparan sulfate proteoglycans occur in plasma membrane, basement membrane, and connective tissue extracellular matrix. These forms differ with respect to their protein composition and chemical characteristics of HS. Information about HS activity in pulmonary diseases is scanty, although HS is a major GAG in the lung. In hypoxia, one of the HS proteoglycans with anticoagulant activity (ryudocan) increases in

cultures of bovine aortic endothelial cells and bovine pulmonary artery endothelial cells (Karlinsky et al. 1992). We found no significant differences in the concentration of HS between control and ozone-exposed animals, however the trend test applied in the analysis clearly indicated significant increases of HS with increasing concentration of ozone. The differences in sulfate contents and the proportions of glucuronic acid to iduronic acid between control and ozone-exposed animals suggest that ozone probably affects the enzymes involved in the epimerization of glucuronic acid to iduronic acid and sulfotransferases involved in the sulfation of HS. Variations in antithrombin III affinity in HS fractions could be attributed to the changes in iduronic acid and sulfation in the antithrombin III binding region of the GAG. Although not studied in this investigation due to limitation of samples, degree of sulfation also affects several other biologic properties of HS, for example, binding to growth factors, cell adhesion, and cell to cell interactions (Jalkanen 1987). In the lung, HS is synthesized mostly by fibroblasts (Sjöberg and Fransson 1977; Vogel and Peterson 1981) and by endothelial cells (Sampson et al. 1975). It is important to determine whether one or both of these cell types are affected by ozone.

In summary, chronic ozone exposure at levels greater than 0.5 ppm causes complex changes in lung connective tissue components. The total concentration of GAG in lung tissues decreases significantly with increasing exposure to ozone. Exposure to ozone decreases the concentration of HA and CS and alters the chemical characteristics of HS. However, whether exposure to 0.12 ppm ozone alters the composition of GAGs is uncertain because the number of observations made in this group were too small to reach conclusions. These observations have implications in the understanding of the damage that ozone causes to the lung structure and function.

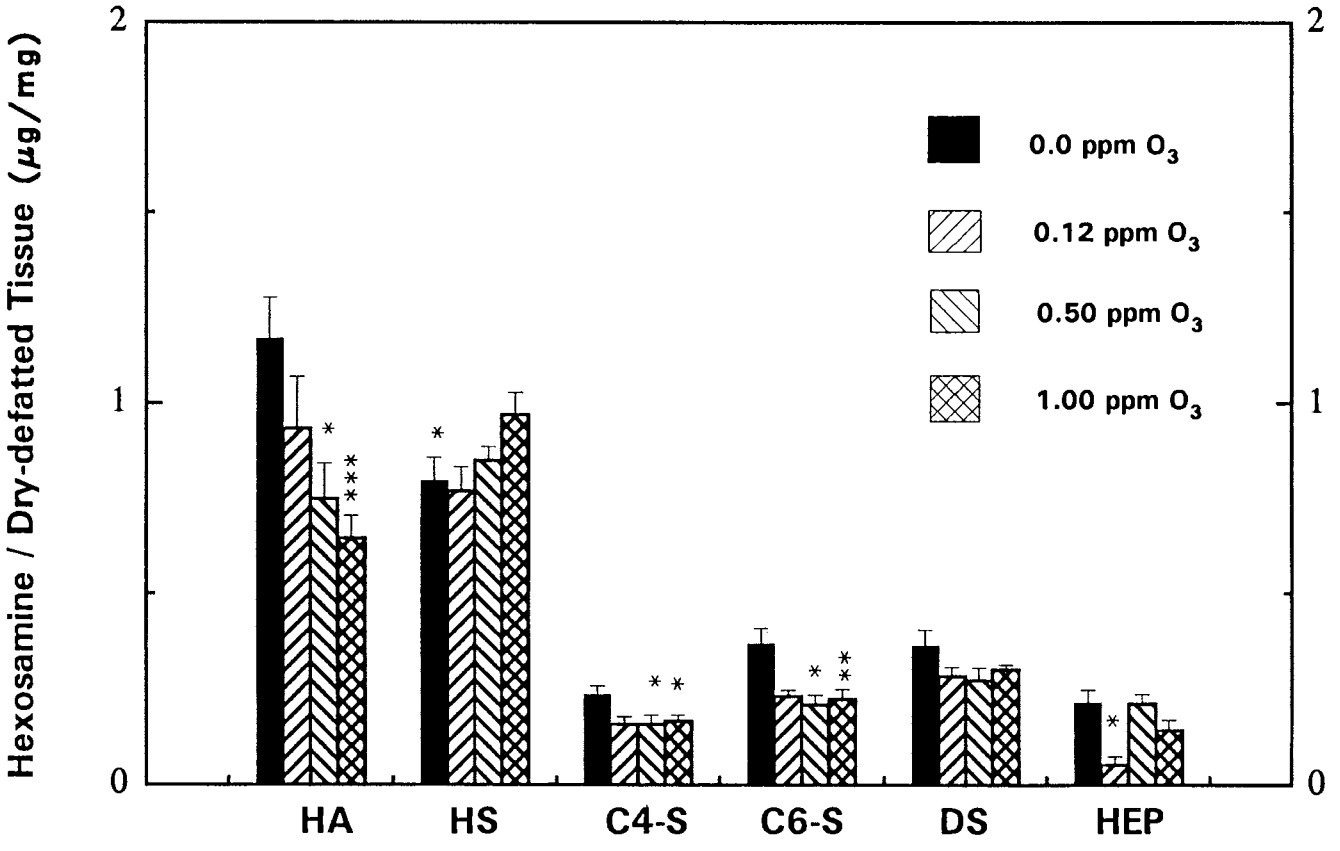

Figure 4. Concentration of individual GAGs from lung tissues of rats exposed to ozone. Fractionation of GAGs was done by HPLC as described in the Methods section. The results are expressed as micrograms of hexosamine per milligram of dry-defatted tissue. Concentrations of individual GAGs were calculated from the amount of hexosamine in pooled fractions under each peak and from the amount of uronate that was fractionated. The p values (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$) shown on ozone groups correspond to pairwise t tests that compare each dose group to the control group. Control group, $n = 10$; exposure to 0.12 ppm ozone, $n = 4$; exposure to 0.5 ppm ozone, $n = 7$; exposure to 1.0 ppm ozone, $n = 10$.

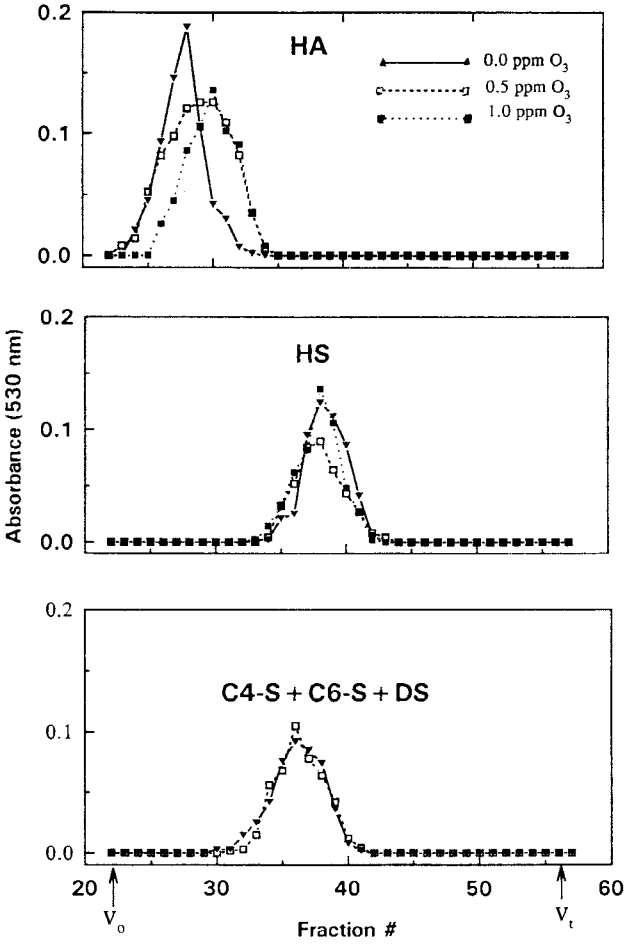

Figure 5. Gel filtration of GAGs from lungs of rats exposed to ozone. Individual GAG fractions from each ozone-exposed group were pooled for gel filtration. Chromatography was performed on a Sepharose CL-2B column (0.9 × 15 cm) as described in the Methods section. The column was monitored for hexosamine. Chondroitin sulfates from the animals exposed to 1.0 ppm ozone were not analyzed because of accidental loss of samples V_o = void volume; V_t = total volume.

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Table 4. Analyses of Heparan Sulfate Fractions from Lung Tissues of Rats Exposed to Ozone[a]

Ozone Exposure	Ratio of Glucuronic Acid to Iduronic Acid[b]	Total Sulfate (mol/mol hexosamine)	*N*-Sulfate (mol/mol hexosamine)	Ratio of Low Affinity to High Affinity of Antithrombin III[c]
0.0 ppm	80:30	1.33	0.34	81.1:18.9
0.5 ppm	92:8	1.16	0.17	90.8:9.2
1.0 ppm	90:10	1.17	0.18	92.0:8.0

[a] All analyses were performed on pooled fractions; thus, there was only one sample in each group.

[b] Uronic acids were quantified by HPLC (Dionex).

[c] Agarose-antithrombin III gel matrix (Sigma) was used to fractionate high-affinity and low-affinity fractions of heparan sulfate. Low-affinity fractions were not retained by the gel matrix and eluted in the wash buffer; high-affinity fractions reacted with the gel matrix and eluted from the column by 2.5 M NaCl.

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ABOUT THE AUTHOR

Bhandaru Radhakrishnamurthy received his Ph.D. degree in chemistry from Osmania University in India in 1958. From 1953 to 1961 he worked at Osmania University as an Assistant Professor in Chemistry. From 1961 to 1992 he was at Louisiana State University School of Medicine in the Departments of Medicine and Biochemistry, where he was Research Associate, Instructor, Assistant Professor, Associate Professor and Professor. From 1992 to the present he has been at Tulane University School of Public Health and Tropical Medicine as a Research Professor in the Department of Applied Health Sciences and at Tulane University School of Medicine as an Adjunct Professor in the Department of Biochemistry. Research interests include connective tissue chemistry, metabolism, and involvement in disease (atherosclerosis, diabetes, chronic obstructive lung disease, and emphysema).

PUBLICATIONS RESULTING FROM THIS RESEARCH

Radhakrishnamurthy B, Berenson GS. 1993. Effect of chronic ozone inhalation on the composition of proteoglycans from rat lungs (Abstract). Glycobiology 3:519.

ABBREVIATIONS

C4-S	chondroitin 4-sulfate
C6-S	chondroitin 6-sulfate
CS	chondroitin sulfates
DS	dermatan sulfate
GAG	glycosaminoglycan
H_2SO_4	sulfuric acid
HA	hyaluronan
Hep	heparin
HEPA	high-efficiency particulate air (filter)
HPLC	high-performance liquid chromatography
HS	heparan sulfate
NaCl	sodium chloride
N-sulfate	sulfate linked to amino nitrogen of glucosamine
SO_4	sulfate

INTRODUCTION

Clinical and epidemiological studies provide evidence that children and young adults exposed to low levels of ozone while exercising for short periods experience transient decrements in lung function (reviewed by Lippmann 1992). It is not known whether repeated or prolonged inhalation of ozone produces long-term decrements in lung function or aggravates existing chronic lung disease. Because exposure to ozone is widespread, and because substantial uncertainties exist regarding the health risks of extended exposure to this pollutant, the National Toxicology Program (NTP) and the Health Effects Institute (HEI) collaborated to evaluate the effects of prolonged exposure to ozone on laboratory animals. (The NTP/HEI collaboration is described in the Introduction to this Research Report.)

In response to RFA 90-1, "Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program–Health Effects Institute Studies: Part B. Structural, Biochemical, and Other Alterations," Dr. Bhandaru Radhakrishnamurthy (then at Louisiana State University School of Medicine, presently at Tulane University School of Public Health and Tropical Medicine) submitted a proposal, "Effects of Ozone Inhalation on Complex Carbohydrates of Lung Connective Tissue." The Health Research Committee approved an 18-month study, which began in July 1991 and cost $195,000. Dr. Radhakrishnamurthy's final report was received at the HEI in May 1993. The revised report was accepted for publication by the Health Review Committee in January 1994.

During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and clarify issues in the Investigator's Report and the Review Committee's Commentary. The following Commentary is intended to serve as an aid to the sponsors of HEI and to the public by highlighting the study's strengths and limitations. Other reports from NTP/HEI Collaborative Ozone Project investigators, including a forthcoming Integrative Summary report, will present biochemical, structural, and functional data and will discuss their implications for human health.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants and other pollutants under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4) through (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants and other pollutants and, in some cases, provide the EPA with limited discretion to modify those requirements.

Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. Ozone's potentially harmful effects on respiratory function led the EPA to promulgate the NAAQS for ozone of 0.12 parts per million (ppm), a level not to be exceeded for more than one hour, once per year. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

The current ozone standard relies heavily on data derived from controlled human exposure studies that demonstrate lung dysfunction within the concentration range of polluted urban air. These studies do not address the issue of potential chronic lung injury that might result from long-term ozone exposures. Because determining the appropriate standards for emissions of oxidants and their precursors depends in part on an assessment of the health risks they present, research on the effects of chronic exposure of the lung to ozone, such as that supported by the NTP/HEI Collaborative Ozone Project, is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Lungs undergo large changes in volume during each respiratory cycle. This unique function requires a specialized structure. For example, the walls of the alveoli must be thin enough to allow the exchange of gases, such as oxygen and carbon dioxide, between blood and cells; yet firm enough to support the alveolar cells; and flexible enough to cope with the changes in lung volume during breathing. The connective tissues that lie between the different cell types of the lung are largely responsible for these properties (reviewed by Juul et al. 1991).

The major components of connective tissues are the fibrous proteins collagen and elastin; proteoglycans, which are composed of proteins that are chemically linked to carbohydrate polymers called glycosaminoglycans; and hyaluronan (hyaluronic acid), the only glycosaminoglycan that is not linked to protein. Collagen and elastin fibers confer tensile strength (the ability to resist the stress of stretching) and elastic recoil properties to the lung. Proteoglycans and hyaluronan fill the spaces between the fibrous proteins and confer structural support. Laboratory evidence suggests that proteoglycans and hyaluronan also influence many cellular events that are critical to lung development and disease, including cell proliferation, migration, and adhesion (reviewed by Juul et al. 1991).

Dr. Radhakrishnamurthy's study focused on ozone's effect on glycosaminoglycans prepared from their naturally occurring proteoglycans, and on hyaluronan. This Background discusses the composition, synthesis, and function of proteoglycans and hyaluronan, and concludes with a short discussion of their changing levels in animal models of pulmonary fibrosis and emphysema.

GLYCOSAMINOGLYCAN AND PROTEOGLYCAN COMPOSITION AND SYNTHESIS

Proteoglycans are composed of one to more than a hundred glycosaminoglycans chemically linked to genetically distinct core proteins. Glycosaminoglycans contain multiple units of carbohydrate molecules that are linked to form a polymer of high molecular weight. A nitrogen-containing molecule, called an amino sugar (either *N*-acetylglucosamine or *N*-acetylgalactosamine), is usually (but not always) linked to a molecule called a uronic acid (either glucuronic acid or iduronic acid). For example, the simplest glycosaminoglycan, hyaluronan, is made up of repeating units of *N*-acetylglucosamine linked to glucuronic acid. Other glycosaminoglycans have sulfate residues linked to oxygen or nitrogen atoms along the polymeric chain. These include chondroitin 4- and 6-sulfates, dermatan sulfate, heparan sulfate, and heparin. Keratan sulfate does not contain a uronic acid; it is composed of sulfated *N*-acetylglucosamine linked to the carbohydrate galactose. The composition of the individual glycosaminoglycans and the tissues in which they are major components are listed in Table 1.

Proteoglycans are synthesized within cells by a complex series of enzymatic reactions: After the core protein is synthesized, a linkage region between the protein and the soon-to-be glycosaminoglycan is formed. First, the carbohydrate xylose is linked to the core protein, followed by two molecules of galactose and a molecule of glucuronic acid, forming the sequence protein-xylose-galactose-galactose-glucuronic acid. Then the components of the glycosaminoglycan are added to the linkage region one by one.

The wide diversity of proteoglycans is derived from the number of different core proteins, the polydisperse nature of the glycosaminoglycans introduced by the biosynthetic process (as seen by variations in molecular size, amino sugar component, sulfate content, and the presence of glucuronic acid or iduronic acid or both), and the various numbers and types of glycosaminoglycans linked to the core proteins (Juul et al. 1991).

Table 1. Composition and Source of Glycosaminoglycans[a]

Glycosaminoglycan	Composition	Linked to Protein	Tissue Distribution[b]
Hyaluronan	Glucuronic acid, *N*-acetylglucosamine	No	Skin, vitreous humor, aorta, heart, cartilage, synovial fluid
Chondroitin 4-sulfate	Glucuronic acid, *N*-acetylgalactosamine, sulfate	Yes	Cartilage, tendon, cornea, bone, skin, arteries
Chondroitin 6-sulfate	Glucuronic acid, *N*-acetylgalactosamine, sulfate	Yes	Aorta, cartilage, cornea, bone, tendon, skin, arteries
Dermatan sulfate	Glucuronic acid, iduronic acid, *N*-acetylgalactosamine, sulfate	Yes	Skin, blood vessels, heart, heart valves
Heparan sulfate	Glucuronic acid, iduronic acid, *N*-acetylglucosamine, sulfate	Yes	Aorta, lung, spleen, liver, kidney
Heparin	Glucuronic acid, iduronic acid, *N*-acetylglucosamine, sulfate	Yes	Lung, liver, skin
Keratan sulfate	Galactose, *N*-acetylglucosamine, sulfate	Yes	Cornea, cartilage, intervertebral disk

[a] Adapted from Radhakrishnamurthy and Berenson (1989).

[b] Tissue distribution indicates the major sources of the glycosaminoglycan.

In the following discussion, proteoglycans are identified by the glycosaminoglycan component linked to the core protein, for example, a heparan sulfate-proteoglycan.

FUNCTIONS OF PROTEOGLYCANS AND HYALURONAN

Proteoglycans and hyaluronan are ubiquitous tissue components. Proteoglycans are found in the extracellular matrix, in granules stored within cells, in basement membranes, and attached to cell surfaces. Hyaluronan is not stored intracellularly but is found in the other milieus.

Extracellular Proteoglycans

Proteoglycans and hyaluronan associate in the extracellular matrix to provide structural support to many tissues and organs. Within the matrix, many (but not all) individual proteoglycans align themselves along the hyaluronan chain to form aggregates of high molecular weight that confer the ability to absorb compressive loads. The association between proteoglycans and hyaluronan can be stabilized by a link glycoprotein that binds to an area of the core protein and to hyaluronan (Heinegård and Oldberg 1989).

Hyaluronan's high molecular weight and the concomitant presence of many glucuronic acid molecules, together with the sulfate and uronic acid present in the glycosaminoglycan chains of proteoglycans, confer strong negative charges to these polymers that attract positively charged ions such as sodium. The osmotic imbalance caused by local high concentrations of sodium ions draws water from surrounding areas to the areas with hyaluronan and proteoglycans. The polymers swell in the aqueous environment and resist compressive forces. The large hydrodynamic volume of hyaluronan and proteoglycans may help maintain the proper water balance in lung tissue thus playing an important role in lung compliance (Takahashi et al. 1983; Juul et al. 1991; Scott 1991; Laurent and Fraser 1992).

Another class of proteoglycans is associated with collagen rather than hyaluronan in the extracellular matrix, and may modulate the formation of collagen fibrils (Kjellén and Lindahl 1991). Small, interstitial dermatan sulfate–proteoglycans have been isolated from bovine lung, and resemble those isolated from other tissues containing fibrillar collagen (van Kuppevelt et al. 1987). The physical association of these proteoglycans with collagen, combined with an ability to increase fibrillogenesis, also could increase the structural support provided by the extracellular matrix.

Basement Membrane Proteoglycans

In addition to their possible role in the lung interstitium, proteoglycans may be important in the organization of lung basement membranes, which are highly specialized extracellular matrix structures that lie beneath epithelial and endothelial cells. Lung basement membranes provide a structural framework for the attachment of these cells, as well as a permeability barrier for the exchange of oxygen and plasma components. These membranes also provide a major barrier to airborne particulates (Juul et al. 1991). The major constituents of basement membranes, type IV collagen (a nonfibrous form of collagen), heparan sulfate–proteoglycan, and the glycoproteins laminin and nidogen, form basement membranes by self-assembly processes and by specific interactions (Timpl 1993). For example, heparan sulfate–proteoglycan binds to laminin via sites in the glycosaminoglycan component, and to nidogen via sites in the core protein (Battaglia et al. 1992). Inhibiting the synthesis of a normal heparan sulfate structure reduces the ability of the heparan sulfate–proteoglycan to bind to laminin. Thus, the presence of a normal heparan sulfate–proteoglycan may be critical for the proper assembly of basement membranes (Brauer et al. 1990).

Intracellular and Cell Surface Proteoglycans

The lungs' defense mechanisms protect them and the rest of the body from infection (Juul 1991). Cells that exert a protective effect in the lungs (neutrophils and macrophages) synthesize proteoglycans and store them within intracellular granules. Heparin, a potent anticoagulant stored within lung mast cells as a small proteoglycan, can exert an anticoagulant effect by binding to antithrombin III, thus forming a better inhibitor of the coagulation agent thrombin than antithrombin III alone (Jackson et al. 1991). Heparin is not the only anticoagulant present in lung tissue. A closely related molecule, the heparan sulfate–proteoglycan from bovine lung, also possesses anticoagulant activity (Radhakrishnamurthy et al. 1980). Indirect evidence (Marcum et al. 1985, 1986) suggests that the receptor for antithrombin III is a heparan sulfate–proteoglycan associated with the endothelial cell surface. These anticoagulant activities may be important in inhibiting blood clotting and platelet aggregation in the lung's extensive capillary bed (Juul 1991).

PROTEOGLYCANS AND HYALURONAN IN CHRONIC LUNG DISEASE

Chronic lung injury can lead to fibrosis and emphysema. Changes in the content of proteoglycans and hyaluronan

often are seen as an early response to injury; the lung's early response may determine the nature and extent of the subsequent dysfunction (Turino 1985; Juul et al. 1991).

Because changes in proteoglycans appear to occur before collagen accumulation in lung fibrosis, it has been speculated that changes in proteoglycans (or hyaluronan) may modulate the later fibrotic response (Juul et al. 1991). Two studies have shown that bleomycin-induced fibrosis in rats and hamsters is characterized by significant increases in the content of lung dermatan sulfate (Phan 1983; Cantor et al. 1983). (To aid in purification and identification, most studies of changes in glycosaminoglycan content use glycosaminoglycans with their core proteins removed.) An increase in glycosaminoglycan content can affect lung mechanics and gas exchange by thickening the alveolar wall, which contributes to the decreased lung compliance associated with fibrotic diseases (Phan 1989).

Increased levels of hyaluronan also have been detected in animal models of fibrosis. Nettelbladt and Hällgren (1989) noted an increased level of hyaluronan in the bronchoalveolar lavage fluid of rats three days after treatment with bleomycin. Nettelbladt and colleagues (1989a) provided histological evidence for an elevated level of hyaluronan in the alveolar interstitial tissue soon after bleomycin was administered to rats. Elevated levels of hyaluronan in the smaller airways, as observed in the early phase of bleomycin-induced injury, also may contribute to interstitial edema by virtue of hyaluronan's ability to bind water (Nettelbladt et al. 1989b) and alter lung compliance. The results of these experimental studies correspond with the results from studies of elevated hyaluronan content in bronchoalveolar lavage fluid from patients with interstitial pulmonary fibrosis (Bjermer et al. 1989). Hyaluronan also is increased in bronchoalveolar lavage fluid from humans with asthma, alveolar proteinosis, sarcoidosis, and adult respiratory distress syndrome (Sahu and Lynn 1978a,b; Hällgren et al. 1985, 1989).

Changes in the content of proteoglycans and hyaluronan have been found in early stages of experimentally induced pulmonary emphysema in laboratory animals (Juul et al. 1991). After inducing emphysema in hamsters by administering elastase, Karlinsky and colleagues (1983) noted that the content of dermatan sulfate–proteoglycan and heparan sulfate–proteoglycan increased after three days, hyaluronan and chondroitin sulfate–proteoglycan increased by day 10, and each returned to the level in control animals by day 21. In contrast, when endotoxin (a complex lipid-carbohydrate polymer) was used to induce emphysema, the synthesis of heparan sulfate remained constant for 72 hours; however, the synthesis of chondroitin sulfate and dermatan sulfate increased at 48 and 72 hours, respectively, before returning to normal levels (Blackwood et al. 1983). These early changes in proteoglycans and hyaluronan may influence cell migration into the damaged area, the cellular response to injury, and the production of elastin and collagen (Juul et al. 1991).

The overall objective of the NTP/HEI Collaborative Ozone Project was to obtain information that could help determine whether prolonged ozone exposure leads to biochemical, structural, or functional changes characteristic of chronic lung disease. Because ozone's effect on hyaluronan or proteoglycans has not been investigated, Dr. Radhakrishnamurthy's study is important for understanding the potential effect of chronic ozone inhalation on these critical complex carbohydrate components of lung connective tissue.

JUSTIFICATION FOR THE STUDY

The Health Effects Institute's primary objective in supporting the set of collaborative studies was to determine whether prolonged ozone inhalation causes changes in the respiratory system of rats that might be related to chronic lung disease in humans. Dr. Radhakrishnamurthy had extensively studied glycosaminoglycan and proteoglycan composition and structure in many tissues, including the lung. To support his proposal, the investigator indicated that the effects of ozone on lung connective tissue fibrous proteins, collagen, and elastin had been examined, but that the effects of ozone on glycosaminoglycans had not. The HEI Research Committee suggested that a study of the effect of long-term ozone exposure on the glycosaminoglycan components of connective tissue would complement the studies of the effects of ozone on lung collagen, which formed part of the collaborative program. These latter studies were performed by Drs. Ling-Yi Chang, Jerold Last, and William Parks.

OBJECTIVES AND STUDY DESIGN

Dr. Radhakrishnamurthy's study was designed to determine whether ozone inhalation altered the content of lung glycosaminoglycans. As described above, all glycosaminoglycans, except hyaluronan, exist physiologically as proteoglycans. Therefore, with the exception of hyaluronan, all of the investigator's results concerning changes in the content of individual glycosaminoglycans should be interpreted as representing changes in the content of their naturally occurring proteoglycans. The study had two major aims:

1. To isolate and quantify the total glycosaminoglycan content of lung lobes from control and ozone-exposed rats.
2. To study changes in the content of the individual glycosaminoglycans as a result of prolonged ozone inhalation.

Although not listed as aims in his proposal, Dr. Radhakrishnamurthy performed additional analyses. He:

1. Compared the molecular size of glycosaminoglycans from control tissue and ozone-exposed tissue;
2. Determined the chemical differences between heparan sulfate isolated from control and ozone-exposed tissue; and
3. Compared the ability of heparan sulfate from control and ozone-exposed tissue to bind to antithrombin III.

The ozone inhalation protocol is described in a Supplement to Part I of Research Report Number 65. Dr. Radhakrishnamurthy obtained the right caudal and accessory lung lobes from rats that had been killed six to eight days after ozone exposure ceased. The core proteins were removed from the sulfated glycosaminoglycans by treating the tissue with proteolytic enzymes and alkali. Removing core protein allows easier separation and identification of the constituent glycosaminoglycans. Next, the tissue extracts containing the sulfated glycosaminoglycans and hyaluronan were analyzed for total and individual glycosaminoglycans.

To quantify the total glycosaminoglycan content in the extract from each lung lobe, the investigator measured the intensity of color that forms when the uronic acid component of glycosaminoglycans reacts with a particular chemical. As mentioned earlier, glycosaminoglycans, with the exception of keratan sulfate, contain uronic acid as one of the repeating carbohydrate molecules. Because lung parenchyma does not contain keratan sulfate, the uronic acid content can be used to measure the content of glycosaminoglycans. However, the investigator encountered a variable interference in the assay for the uronic acid component.

Next, to quantify the levels of individual glycosaminoglycans, Dr. Radhakrishnamurthy separated the glycosaminoglycans from each other by high-performance liquid chromatography. This procedure produced four fractions: hyaluronan, heparan sulfate, a mixed fraction (chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate), and heparin. Because of the interference in the uronic acid assay, the investigator substituted a colorimetric analysis that measures the amino sugar content and thereby quantifies the levels of individual glycosaminoglycans in the extracts from each lung lobe. For the mixed fraction, the investigator determined the levels of chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate using a combination of colorimetric and enzymatic analyses. First, he measured the total amino sugar content in this fraction. Next, with the use of specific degradative enzymes, he determined the proportions of each glycosaminoglycan in this fraction and assigned a corresponding value for amino sugar.

The investigator used gel filtration, a technique that separates molecules on the basis of their size, to compare the molecular sizes of the glycosaminoglycans from control tissues with those from ozone-exposed tissues. Dr. Radhakrishnamurthy also further analyzed the heparan sulfate fraction produced by high-performance liquid chromatography. Heparan sulfate is characterized by the presence of both glucuronic and iduronic acids, and by sulfate bound to oxygen and nitrogen atoms in glucosamine and to an oxygen atom in iduronic acid. Dr. Radhakrishnamurthy used chemical analyses to determine the total amount of sulfate, the amount of sulfate bound to the nitrogen atom in glucosamine, and the amounts of glucuronic and iduronic acids in heparan sulfate prepared from control and ozone-exposed lung lobes. He also compared the ability of heparan sulfate from control and ozone-exposed tissues to bind to antithrombin III by adding solutions of this glycosaminoglycan to a solid matrix containing antithrombin III and then analyzing the degree to which the heparan sulfate preparations bound to the matrix.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The two major objectives of the study were attained. The additional studies of chemical and structural changes in glycosaminoglycans provided a greater insight into ozone's effects than could be gained from measuring changes in content alone.

ASSESSMENT OF METHODS AND STUDY DESIGN

The inhalation component of this project was conducted in compliance with the NTP laboratory health and safety regulations, and with the Food and Drug Administration Good Laboratory Practice Regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

Standard methods were used to extract glycosaminoglycans from lung tissue, to measure glycosaminoglycan content, and to separate and quantify individual glycosaminoglycans. The investigator used reliable chemical and biochemical

techniques to determine structural and chemical differences between glycosaminoglycans from control and ozone-exposed tissue.

STATISTICAL METHODS

Like the study design, the use of statistical analyses also was straightforward. Dr. Radhakrishnamurthy used one-way analysis of variance and multiple linear regression analysis to study ozone's effect on the glycosaminoglycan content of lung tissue. Both this analysis and Dunnett's *t* test were used appropriately. Because changes in heparan sulfate content as a response to ozone were not statistically significant, the investigator applied a trends test to the data using a linear regression analysis to determine the trend in these changes. The test for linear trend is the best initial test for a dose-response relation because this one-degree-of-freedom test is more powerful than comparing any single exposure group to the control group. Because the test for trend combines data over all exposure groups, it is more powerful than a pairwise comparison.

RESULTS AND INTERPRETATIONS

Dr. Radhakrishnamurthy determined that hyaluronan is the predominant glycosaminoglycan in lung lobes from control F344/N rats, accounting for 37% of the total glycosaminoglycan, and heparan sulfate accounted for 25%. The remaining glycosaminoglycans present in smaller amounts were chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and heparin.

A major finding of this study was that lung lobes from rats exposed to 0.5 or 1.0 ppm ozone for 20 months showed statistically significant decreases in total glycosaminoglycan content compared with tissue from control rats. No differences from control rats were seen after exposure to 0.12 ppm ozone.

After separating the individual glycosaminoglycans by high-performance liquid chromatography, Dr. Radhakrishnamurthy found that, compared with control rats, the levels of hyaluronan, chondroitin 4-sulfate, and chondroitin 6-sulfate were reduced significantly in tissue from rats exposed to 0.5 or 1.0 ppm ozone, but that those levels were unchanged in tissue from rats exposed to 0.12 ppm. Because fewer rats were exposed to 0.12 ppm ozone than to the other ozone levels, Dr. Radhakrishnamurthy obtained tissue from only 6 rats exposed to 0.12 ppm in contrast to 14 rats exposed to 0 ppm, 13 rats exposed to 0.5 ppm, and 14 rats exposed to 1.0 ppm ozone. It is possible that changes at a level of 0.12 ppm may have become evident if a larger sample size had been available to the investigator.

Most studies of chemically induced fibrosis and emphysema in laboratory animals have shown that the content of lung proteoglycans (or the sulfated glycosaminoglycans prepared from them) and hyaluronan increased compared with the levels found in control animals. In this study, the major effect of exposing rats to a highly reactive gas such as ozone was a decreased content of these connective tissue components; thus, the effect of ozone on lung proteoglycan and hyaluronan content differs in mechanism from the effects of chemicals such as bleomycin, elastase, and endotoxin used to induce lung fibrosis or emphysema in laboratory animals.

It is not known whether the reduction in levels of hyaluronan and the chondroitin 4- and 6-sulfate proteoglycans was caused by a decreased synthesis of the glycosaminoglycan or, in the case of the proteoglycans, a decreased synthesis of the core protein portion of the molecule. Oxidants inactivate proteinase inhibitors (Johnson and Travis 1979); therefore, increased proteinase activity also could have caused lower levels of the proteoglycans. Because the body weights of control and ozone-exposed rats did not differ, impaired nutrition did not play a role in decreasing glycosaminoglycan levels. In addition, serum from sentinal rats in the NTP study tested negatively for a variety of respiratory pathogens, suggesting that respiratory infection did not influence the results.

Dr. Radhakrishnamurthy observed that the hyaluronan isolated from tissues exposed to 0.5 or 1.0 ppm ozone was smaller in molecular size than that isolated from control tissues. In contrast, neither heparan sulfate nor the mixture of chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate was affected. These results suggest that ozone specifically degraded hyaluronan.

Decreases in hyaluronan and in the chondroitin 4-sulfate and chondroitin 6-sulfate proteoglycans could have important consequences on lung function by weakening the support conferred by the extracellular matrix. In addition, because the molecular size of hyaluronan in ozone-exposed tissue is smaller than in normal tissue, hyaluronan may have a reduced ability to aggregate proteoglycans, an effect that also could weaken the supportive function of the matrix.

In contrast to the results with hyaluronan and chondroitin 4- and 6-sulfates, the content of heparan sulfate increased at the two highest ozone concentrations. Although these differences were not statistically significant when analysis of variance and linear regression were applied, a trends test applied to the data showed a significant trend of increasing heparan sulfate at ozone levels of 0.5 and 1.0 ppm. Figure 4 of the Investigator's Report indicates that

heparan sulfate replaced hyaluronan as the predominant glycosaminoglycan in tissue from rats exposed to 0.5 and 1.0 ppm ozone.

The heparan sulfate from lung tissues exposed to 0.5 and 1.0 ppm ozone differed in chemical characteristics from control tissues. The proportion of iduronic acid to glucuronic acid, the total amount of sulfate, and the amount of sulfate bound to the nitrogen atom of glucosamine were reduced in heparan sulfate from ozone-exposed tissues. Finally, heparan sulfate from ozone-exposed tissues had a lower affinity for antithrombin III than that from control tissue.

The significance of the apparent increase in heparan sulfate resulting from ozone exposure is not clear. However, the decreased sulfate and iduronic acid content in heparan sulfate from ozone-exposed tissues may have been responsible for its decreased binding affinity for antithrombin III. A study of the closely related glycosaminoglycan heparin has shown that sulfate and iduronic acid are critical for binding to antithrombin III (Jackson et al. 1991).

The content of heparin decreased significantly at 0.12 ppm ozone, but not at the higher ozone levels. However, the number of animals in the affected group was too small to draw conclusions from this observation. No change in dermatan sulfate from control tissues was seen at any ozone concentration.

IMPLICATIONS FOR FUTURE RESEARCH

Measurements of changes in extracellular matrix components are best performed over a broad time span, in conjunction with other connective tissue measurements, and should include morphological correlations. These criteria provide a context for discussing specific changes and permit some degree of meaningful speculation. The study design mandated by the NTP protocol precluded serial measurements; therefore, this study measured ozone-induced changes in hyaluronan and proteoglycan content at only one time point. However, the results from studies of matrix proteins, which were conducted by Drs. Last, Parks, and Chang as part of the NTP/HEI Collaborative Ozone Project, should help when interpreting Dr. Radhakrishnamurthy's findings.

Because Dr. Radhakrishnamurthy's study did not explore the mechanism by which ozone affects lung glycosaminoglycan content, future research should be directed toward determining the factors responsible for these effects. In vitro culture studies of cells that synthesize hyaluronan and proteoglycans (fibroblasts and endothelial cells), prepared from unexposed and ozone-exposed lung tissue, could demonstrate whether their viability or ability to synthesize hyaluronan or proteoglycans are affected. Investigation of protease inhibitor activity could indicate whether enhanced degradation is involved in causing decreased levels of proteoglycans.

Future research also should focus on determining the overall importance of alterations in hyaluronan or proteoglycan composition on the structure and function of the lung. For example, does altering hyaluronan and proteoglycan composition lead to permanent structural damage to the lung parenchyma, or is the initial damage a temporary change? Are the physical properties or synthesis of other matrix components, such as collagen and elastin, affected by alterations in hyaluronan and proteoglycan composition? Do such alterations affect lung fluid movement, permeability, or growth and development? These are critical issues; finding the answers may be influential in determining the human health effects of ozone exposure.

CONCLUSIONS

This study has made an important contribution to our understanding of ozone's effects on lung connective tissue. The effects of ozone on lung collagen and elastin have been studied in laboratory animals; however, this is the first report on the effect of ozone on the complex carbohydrate components of connective tissue.

The total glycosaminoglycan content of lungs from rats exposed to 0.5 or 1.0 ppm ozone was decreased compared with lungs from control rats breathing filtered air. No effect was seen at 0.12 ppm ozone (although the sample size at this ozone level was markedly smaller than at the higher levels). When glycosaminoglycans were separated into individual components, the content of hyaluronan, the predominant glycosaminoglycan in control rat lung, was decreased in lungs from rats exposed to 0.5 or 1.0 ppm ozone, as was the content of two minor rat lung glycosaminoglycans, chondroitin 4-sulfate and chondroitin 6-sulfate. Again, no effects were seen at 0.12 ppm ozone.

In contrast to the results with hyaluronan, chondroitin 4-sulfate, and chondroitin 6-sulfate, the content of heparan sulfate, the second major glycosaminoglycan in normal rat lung, showed a significant trend toward increased levels in tissue from rats exposed to 0.5 and 1.0 ppm ozone, compared with control rats. No effects were seen at an ozone

level of 0.12 ppm. The content of dermatan sulfate and heparin did not change significantly from control values at any level of ozone.

The molecular size of hyaluronan was lower in tissue from rats exposed to 0.5 or 1.0 ppm ozone, possibly due to oxidative degradation by this highly reactive gas. This degradative effect appeared to be specific for hyaluronan; neither heparan sulfate nor a mixed preparation of chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate were affected.

Ozone-induced chemical changes in heparan sulfate also were noted. The sulfate content, the amount of sulfate bound to the nitrogen atom of glucosamine, and the amount of iduronic acid were lower in heparan sulfate prepared from rats exposed to 0.5 and 1.0 ppm ozone than in that from control lung lobes. These changes may have been responsible for the decreased ability of heparan sulfate from ozone-exposed lung tissue to bind to antithrombin III, one mechanism by which heparan sulfate and heparin may exert their anticoagulant effect.

The reason for the apparent increase in one type of proteoglycan (the heparan sulfate proteoglycan), when the content of two others (the chondroitin 4-sulfate and chondroitin 6-sulfate proteoglycans) decreased, is unclear. However, the lower content of hyaluronan and two proteoglycans, together with a decreased molecular size of hyaluronan, may impair lung function by changing the properties of the lung's extracellular matrix.

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